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Secreted Apolipoprotein E Reduces Macrophage-Mediated LDL Oxidation in an Isoform-Dependent Way

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Abstract As an inflammatory cell, the macrophage produces various oxidizing agents, such as free radical species. These can modify LDL as a secondary effect and doing so may favor atherogenic processes. Any molecule able to counteract these reactions would be of much benefit, especially if secreted by the macrophage itself at the lesion site. Such is the case for apolipoprotein E (apoE), which has been shown to exert antioxidant properties in some studies, mostly in relation to Alzheimer's disease. In this study, we assessed the antioxidant potential of the various isoforms of apoE (E2, E3, and E4) using a metal-induced LDL oxidation system with exogenous recombinant apoE and an in vitro model of macrophage-mediated LDL oxidation. We found that all three isoforms had an antioxidant capacity. However, whereas apoE2 was the most protective isoform in the cell-free system, the opposite was observed in apoE-transfected J774 macrophages. In the latter model, cellular cholesterol efflux was found to be more important with apoE2, possibly explaining the larger quantity of oxidative indices observed in the medium. It is proposed that the antioxidant property of apoE results from a balance between direct apoE antioxidant capacities, such as the ability to trap free radicals, and potentially pro-oxidative indirect events associated with cholesterol efflux from cells. Our observations add to the therapeutic potential of apoE. However, they also suggest the need for more experiments in order to achieve careful selection of the apoE isoform to be targeted, especially in the perspective of apoE transgene use. J. Cell. Biochem. 90: 766–776, 2003.

Key words: apoE; LDL oxidation; antioxidant; macrophage; cholesterol; atherosclerosis

It is well known that high circulating levels of LDL are strongly associated with atherosclerosis. However, it has become clear that post-secretory modifications of LDL also contribute to their atherogenicity [Witztum and Steinberg, 2001]. Oxidative modification of LDL is probably the most important and is widely regarded as a critical event in the atherogenic process [Steinbrecher et al., 1990; Witztum and Steinberg, 1991]. The oxidation hypothesis suggests that as LDL becomes oxidized, the lipoprotein loses its ability to be recognized by the LDL receptor and increases its affinity for scavenger receptors on monocytes, smooth muscle cells, and macrophages in the arterial intima. Unlike the LDL receptor, scavenger receptors are not regulated by intracellular concentrations of cholesterol [Steinberg et al., 1989; Witztum and Steinberg, 1991]. Consequently, the uptake of oxidized LDL results in the formation of lipid-laden foam cells, characteristic of fatty streaks that are the early lesions of atherosclerosis, still reversible [Steinberg et al., 1989].

Macrophages have been reported to be the source of various oxidizing agents able to modify LDL, such as free radical species. Conversely, they also secrete components with potential

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antioxidant properties. This is the case for apolipoprotein E (apoE). ApoE is a 299-amino acid polypeptide whose primary function is the receptor-mediated clearance of lipoproteins and cholesterol redistribution among cells. Three common isoforms exist in humans. These differ in only two amino acid positions and are designated E2, containing cysteine at residues 112 and 158, E3 with cysteine at 112 and arginine at 158, and E4 with arginine at both sites [Mahley, 1988; Mazzone, 1996]. These isoforms are encoded for by the alleles termed ε2, ε3, and ε4 with a frequency in Caucasian populations of 8, 77, and 15%, respectively. This polymorphism affects the risk of both coronary artery disease (CAD) and Alzheimer's disease, both of which are more common in persons expressing the E4 isoform [Davignon et al., 1999]. The isoforms differ from each other in their binding affinity for apoE receptors thus affecting plasma lipoprotein levels. For a long time, this effect of apoE has been its only known association with the variation in CAD risk.

Macrophages produce apoE, although to a much lesser extent than do hepatocytes [Mazzone, 1996]. Transplantation of wild-type bone marrow [Boisvert et al., 1995; Linton et al., 1995] or selective expression of a human apoE transgene in macrophages [Bellosta et al., 1995; Boisvert et al., 1995] decreased atherosclerosis in apoE-deficient mice. Conversely, on a Western diet, atherosclerosis was promoted in wild-type mice receiving bone marrow from apoE-deficient mice [Fazio et al., 1997]. Because expression of a human apoE transgene in macrophages of apoE-deficient mice [Bellosta et al., 1995] and transplantation of apoE-deficient macrophage stem cells into wild-type mice [Fazio et al., 1997] produced little changes in plasma lipid levels, it is believed that macrophage-derived apoE exerts antiatherogenic properties largely independent of its effects on plasma lipoproteins. One possible explanation for the antiatherogenicity of macrophage-derived apoE is its contribution to cellular cholesterol efflux from these cells [Mazzone, 1996; Davignon et al., 1999]. Another explanation concerns the antioxidant properties of apoE. For the last few years, apoE has been shown to exhibit antioxidant effects mainly in relation to Alzeimer's disease. To our knowledge, few studies focusing on the antioxidant role of apoE in relation to atherogenesis have been published.

The aim of this study was thus to assess the antioxidant potential of apoE in an in vitro model of macrophage-mediated LDL oxidation and to relate this action to the various isoforms of apoE (E2, E3, and E4).

MATERIALS AND METHODS

LDL Isolation

LDL ($1.019 \leq \text{density} \leq 1.063$) was isolated by sequential ultracentrifugation from human pooled plasma samples (stored at $-70^{\circ}C$ in the presence of sucrose [Havel et al., 1955; Rumsey et al., 1994; Mabile et al., 2001]. LDL samples were dialyzed against PBS containing 0.3 mM EDTA, sterilized by filtration (0.4 μM Millipore filters), and stored at 4°C under nitrogen until use (within 1 week). Before experiments, LDL was dialyzed against EDTA-free PBS and the protein content was measured using bovine albumin as the standard [Lowry et al., 1951].

Cell Culture

Murine apoE-deficient J774 macrophages were obtained from the American Type Culture Collection (Rockville, MD). Cells were seeded in six-multiwell plates and grown in Dulbecco's modified Eagle medium (DMEM) containing 100 μM non-essential amino acids, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% fetal calf serum (FCS), in a humidified incubator (5% CO_2, 37°C).

J774 Cells Transfection With ApoE

Stably transfected J774 cells were prepared by transfection of the expression vector $pcDNA_3$ containing the cDNA of apoE2, E3, or E4. cDNAs for the three isoforms of apoE were a generous gift from Dr. Karl Weisgraber (Gladstone Institute of Cardiovascular Disease, San Francisco, CA). The signal sequence was first added by PCR using appropriate primers. All cDNAs were sequenced on an Applied Biosystems 373A DNA sequencer using a Thermo Sequenase dye terminator kit (Amersham Pharmacia Biotech, Baie D'urfé, Que.), before subcloning into the mammalian expression vector $pcDNA_3$. Transfection of the three apoE-containing vectors and of the vector only (control) was performed using Lipofectin reagent (Gibco, Burlington, Canada). Stable transfectants were selected on DMEM 10% FCS supplemented with 400 μg/ml geneticin (G418). In order to select clones secreting similar amounts of apoE, levels of apoE2, E3, or E4 in

the medium were determined by sandwich ELISA [Cohn et al., 1996].

ApoE Secretion

Subconfluent J774 macrophages were cultured for 24 h in DMEM free of FCS in order to exclude an exogenous source of apoE, washed and cultured for a further 24 h in RPMI-1640 medium (RPMI medium containing less phenol-red than DMEM medium thus avoiding any interference with the assay). This medium was collected, centrifuged to remove cell debris and analyzed for apoE secretion by sandwich ELISA [Cohn et al., 1996].

Preparation of Recombinant ApoE

Recombinant apoE2, E3, and E4 were prepared according to the method of Biotech Pharmacia, Baie D'urfé, Que. (bulk GST purification module). cDNAs were inserted in frame into the pGEX-6P-3 vector. Positive clones were induced by 0.5 mM IPTG for 45 min. Bacteria were collected by centrifugation at 3,500 rpm for 20 min and lysed by sonication in 1× PBS + 0.1% Triton + protease inhibitors. The fusion protein was bulk purified on Gluthatione Sepharose 4B. The GST moiety was removed by cleavage with Pre Scission© protease (Amersham). The yield was around 0.5 mg of fusion protein/L bacterial culture.

LDL Oxidation

J774 cells (transfected or not) were grown as described in the previous section until subconfluence was reached, washed twice with PBS, and incubated for 16 h with 150 μg LDL in 1 ml of Ham's F10 medium (containing trace amounts of iron and copper). Oxidation was stopped by addition of EDTA (5%) and BHT (0.2%) followed by storage at −70°C.

LDL (50 μg) was oxidized by $CuSO_4$ (10 μM) or AAPH (2,2′-azobis(2-amidinopropane)dihydrochloride), a free radical generator (1 mM) in sterile PBS (1 ml) for various times. Oxidation was stopped by addition of EDTA (5%) and BHT (0.2%), followed by storage at −70°C.

Evaluation of LDL Oxidation

Lipoperoxidation was evaluated by the Thio-Barbituric Acid Reactive Substances (TBARS) assay adapted from the method of Yagi [1987]: 0.3 ml of the test medium containing control or modified LDL was incubated with 0.7 ml of TBA/TCA (0.375%/15%) at 100°C for 30 min. The absorbance was read at 535 nm versus standards of tetramethoxypropane (Sigma, Toronto, Canada). Further, the oxidation of LDL lipids was assessed by measuring the consumption of the polyunsaturated fatty acids (PUFAs), linoleic acid (LA), and arachidonic acid (AA) [Sattler et al., 1991]. After saponification, acidification, and methylation of the medium samples, fatty acids were analyzed by gas chromatography (Innowax column, Hewlett-Packard, L = 30 m, internal diameter = 0.25 mm). Results are expressed as percentage of total fatty acids. In the AAPH-mediated LDL oxidation model, lipoperoxidation occurred within a few hours and was monitored by measuring the increased formation of conjugated dienes by recording their absorbance at 234 nm every 20 min for 5 h [Esterbauer et al., 1989]. For each oxidation curve, the lag time was determined graphically. LDL protein oxidation was evaluated by measuring the electrophoretic mobility on agarose gel electrophoresis (Beckman Paragon System, Toronto, Canada).

Cell Cholesterol Measurement

After 16-h incubation with 150 μg of LDL in 1 ml of Ham's F10 medium, apoE-transfected cells were washed and placed in the efflux medium (DMEM + 0.1% BSA) for 1, 3, 8, or 16 h more. At each time point, aliquots of medium were collected from which lipids were extracted with hexane/isopropanol 3/2 v/v, saponified with KOH 0.5 M/MeOH; saponified products were extracted with hexane, derivatized with DMFO (dimethylformamide) and BSTFA (bis(trimethylsilyltrifluoroacetamide, 4:1) and injected into the SPB-1701 column (Supelco, Oakville, Canada, L = 30 m, diameter = 0.25 mm) for gas chromatography (bonded phase: 14% cyanopropylphenyl/86% dimethylsiloxane) and total cholesterol measurement. After their 16-h incubation with LDL, cell total cholesterol was evaluated similarly in the transfected cells, after lysis in the following buffer: Tris-base 50 mM, pH 7.4, KCl 150 mM, and Triton X-100 1%.

Protein Measurement

Cell protein was measured according to the method of Lowry et al. [1951] after two washes with PBS and lysis in the buffer described above.

Statistical Analysis

Data are expressed as means ± SD. Statistical analyses were performed using unpaired

Student's *t*-tests. Threshold of significance was $P < 0.05$.

RESULTS

ApoE2, E3, or E4-transfected cell clones were selected on their capacity to secrete apoE at physiological levels (1 μg/mg cell protein) (Table I). The following clones were used for all the experiments reported in the present article: apoE2/15, apoE3/16, and apoE4/3.

Effect of Secreted ApoE on LDL Oxidation by Macrophages

LDL was oxidized in Ham's F10 medium by the apoE-transfected J774 macrophages secreting 1 μg/ml of either apoE2, E3, or E4. The LDL oxidation rate was evaluated by measuring, in the LDL-containing medium, the consumption of LA and AA, respectively, the formation of TBARS and by following the modification of the LDL electrophoretic pattern (Fig. 1). As evidenced in the three assays, oxidation of LDL by apoE-secreting cells was significantly lower than oxidation of LDL by control cells (transfected by the vector only). Specifically, apoE3 and apoE4 reduced the oxidation of LA and AA to a similar extent in contrast to apoE2 whose protective effect was less marked (Fig. 1A,B). These results were corroborated by the levels of TBARS produced over the period of incubation (Fig. 1C). Likewise, the electrophoretic mobility (Fig. 1D) of the oxidized LDL was consistent with the corresponding amount of TBARS (Fig. 1C). Taken together, these results suggest that secreted apoE can exert antioxidant properties whatever the isoform and that apoE3 and apoE4 provide a more effective protection against oxidation than apoE2.

TABLE I. Levels of ApoE Secreted by Transfectants

Transfected J774 clones	Secreted apoE (ng/mg cell protein)
Control vector	0
ApoE2 clone	
1	9
11	54
15	984
8	1,932
7	4,185
ApoE3 clone	
1	7
13	80
16	968
3	1,426
ApoE4 clone	
13	10
1	161
15	304
10	877
3	1,025

ApoE-deficient J774 macrophages were transfected with pcDNA3 containing either apoE2, apoE3, or apoE4 cDNAs. Stable clones were selected and apoE concentrations were measured in the medium.

LDL Oxidation by Free Radicals in the Presence of Recombinant ApoE

In an attempt to investigate the potential antioxidant properties of apoE ex vivo, we first examined the ability of the three isoforms to interfere with free radical-mediated oxidation. Oxidation of LDL (150 μg) by AAPH (1 mM), in the presence of recombinant apoE (1, 10, 25, and 50 μg/ml), was studied by monitoring the formation of conjugated dienes and by determining the oxidation lag time (Fig. 2 and Table II). At concentrations of 25 and 50 μg/ml, all apoE isoforms significantly retarded LDL oxidation by twofold. No significant difference was seen between the three isoforms at any of the doses tested (conjugated diene formation kinetic curves shown for 25 μg/ml on Fig. 2). Thus, apoE was able to scavenge free radicals and, in our model, this process was not isoform dependent.

LDL Oxidation by Copper in the Presence of Recombinant ApoE

Cell-induced oxidative processes can also be initiated by transition metals and apoE has been reported to inhibit metal-induced oxidation of lipoproteins in an allele-specific way [Miyata and Smith, 1996]. We sought to determine whether this occurred in our model and whether it could explain the isoform specificity. LDL (150 μg) was, therefore, oxidized by $CuSO_4$ (10 μM) in the presence of various concentrations of recombinant apoE2, E3, or E4. Oxidation was evaluated by measurement of TBARS formation (Fig. 3A) and LDL electrophoretic mobility (Fig. 3B). The degree of oxidation was reduced in a dose-dependent way by all apoE isoforms (not shown). At a concentration of 25 μg/ml, apoE2 was the most potent in protecting LDL, apoE3 had an intermediate effect and apoE4 was the least effective (Fig. 3A). This observation was confirmed by electrophoresis (Fig. 3B). At a concentration of 50 μg/ml, inhibition was complete with all of the isoforms (data not shown). Thus, apoE was able to reduce metal-induced oxidative events and the extent to which this occurred depended on the

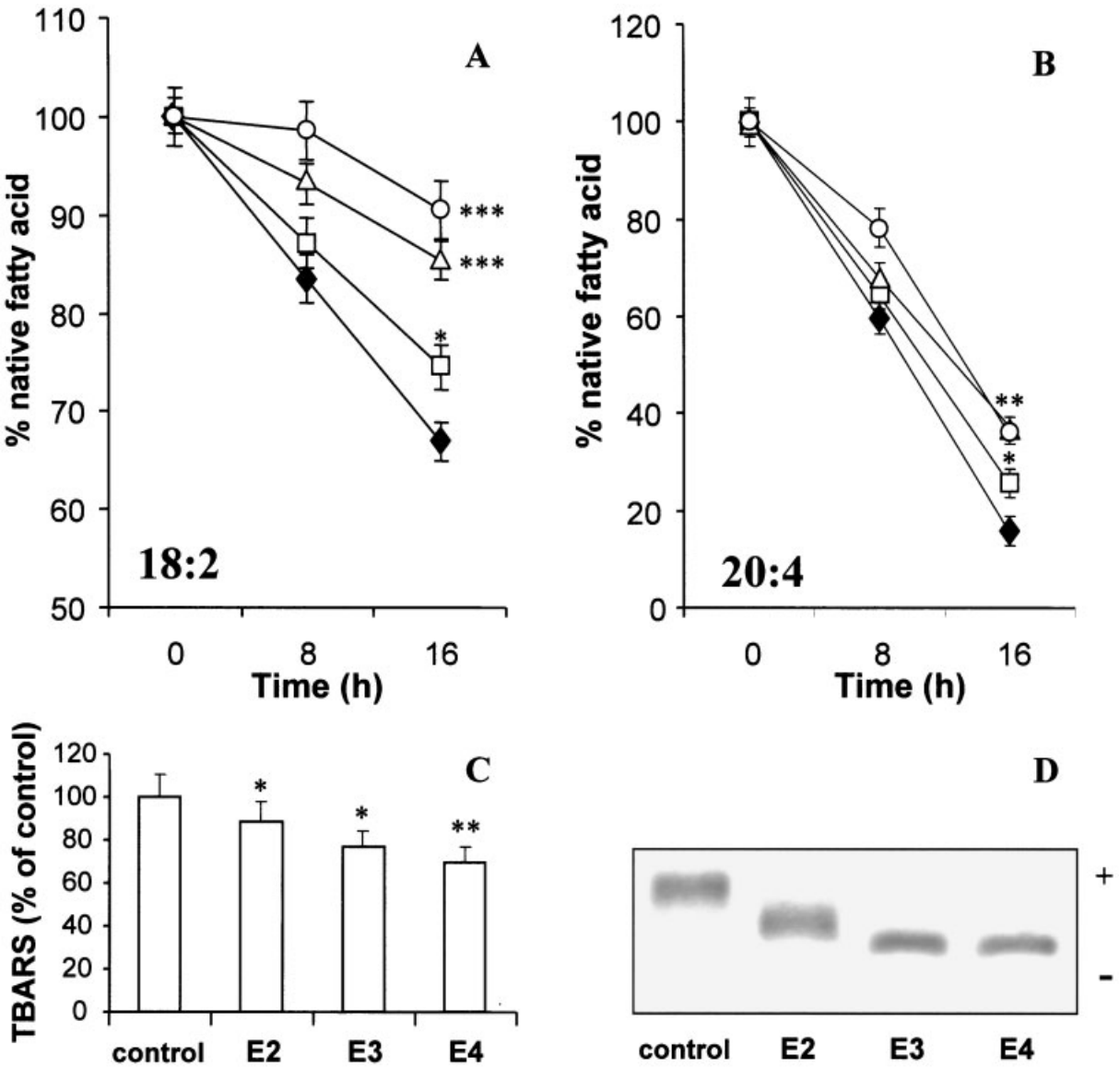

Fig. 1. Effect of apolipoprotein E (apoE)2, E3, and E4 on LDL oxidation by apoE-transfected J774 macrophages. One hundred fifty micrograms of LDL were incubated in 1 ml Ham's F10 medium for 16 h. Oxidation of LDL was evaluated by measuring consumption of linoleic acid (LA, **A**) and arachidonic acid (AA, **B**), formation of ThioBarbituric Acid Reactive Substances (TBARS, **C**), and electrophoretic mobility of LDL, as presented for one representative experiment (**D**). Black diamonds: control (oxidized by apoE-deficient macrophages), squares: apoE2, triangles: apoE3, circles: apoE4. Data are means ± SD of six independent experiments. Statistical difference from control: $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

isoform. However, these results did not show the same isoform-specificity observed in the cell-mediated oxidation model.

Differential Effect of ApoE Isoforms on Cholesterol Efflux

ApoE has been shown to efflux cholesterol in an isoform-specific way [Cullen et al., 1998; Curtiss and Boisvert, 2000]. As cholesterol is a good substrate for oxidation, it could account for increased oxidation indices in the medium. Thus, we studied whether cholesterol was distinctly effluxed from the transfected macrophages as a function of the isoform. Accordingly, apoE2-, apoE3-, or apoE4-transfected J774 cells were tested for their ability to secrete cholesterol over and after the 16 h period of incubation with LDL (required for the LDL oxidation and leading to the subsequent load of oxidized LDL within the cell, as shown in Table III). Cell cholesterol was measured at the end of 16 h (Table III) and in the efflux medium after a

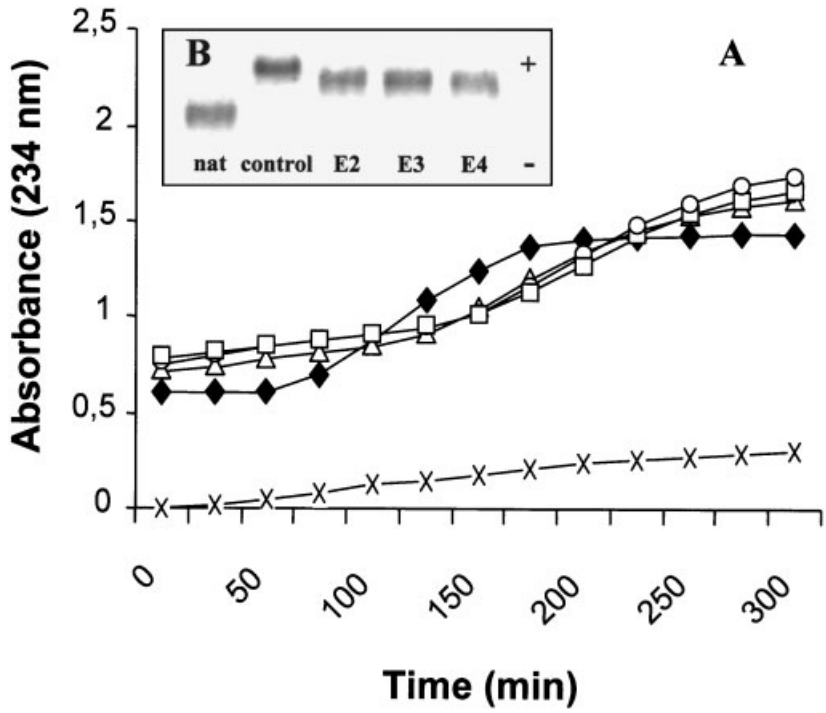

Fig. 2. Effect of exogenous recombinant apoE2, E3, and E4 on LDL oxidation by the free radical-generator AAPH. One hundred fifty micrograms of LDL were incubated with 1 mM AAPH in 1 ml PBS for 5 h. Conjugated diene generation was monitored every 25 min by measuring increase in absorbance at 234 nm, as shown for one representative experiment after the addition of 25 μg/ml apoE (**A**). Crosses: native (nat) LDL, black diamonds: oxidized control, squares: apoE2, triangles: apoE3, circles: apoE4. The electrophoretic mobility of LDL after 200 min of oxidation in the presence of the various apoE isoforms (25 μg/ml) is shown for one representative experiment (**B**). Data are means ± SD of four independent experiments.

further 1, 3, 8, and 16 h of incubation (Fig. 4). At the end of the 16 h incubation with LDL, total cell cholesterol was significantly higher in control cells than in apoE-expressing cells partly due to the apoE ability to efflux cholesterol out of the cell (Table III). Furthermore, intracellular cholesterol was significantly lower in the apoE2-expressing cells than in those expressing apoE3 or E4, suggesting that apoE2 was more efficient at effluxing cholesterol than the two other isoforms. Accordingly, after 16 h, the medium from apoE2-transfected macrophages contained significantly more cholesterol than the medium from apoE3- or E4-transfected cells (Fig. 4).

TABLE II. Lag Time of Conjugated Diene Generation in LDL Following Incubation With AAPH in the Presence of Recombinant ApoE2, ApoE3, or ApoE4, or in the Absence of ApoE (Control)

	Lag time		
Added apoE	10 μg/ml	25 μg/ml	50 μg/ml
Control (none)	65 ± 15	61 ± 15	60 ± 18
ApoE2	75 ± 16	115 ± 18*	135 ± 13*
ApoE3	67 ± 10	120 ± 13*	125 ± 14*
ApoE4	78 ± 19	122 ± 20*	130 ± 12*

AAPH, 2,2′-azobis(2-amidinopropane)dihydrochloride.
Data are from three separate experiments and are expressed as means ± SD.
*$P < 0.001$, significantly different from control.

DISCUSSION

LDL oxidation is assumed to occur in the intimal space of the arterial wall where it gathers various elements favoring oxidative processes. It is an antioxidant-depleted area [Steinberg, 1997] containing various inflammatory cells such as macrophages [Hansson et al., 1989] and pro-oxidant molecules such as reactive oxygen species and transition metals (Fe^{3+}, Cu^{2+}), at lesion-prone sites [Stocker, 1994]. Any molecule capable of preventing or reducing oxidative reactions could contribute to the inhibition of LDL oxidation and, by extension, to slowing down atherogenesis. ApoE has been shown to be secreted by macrophages on the one hand, and to have antioxidant properties, on the other hand, but this has been studied

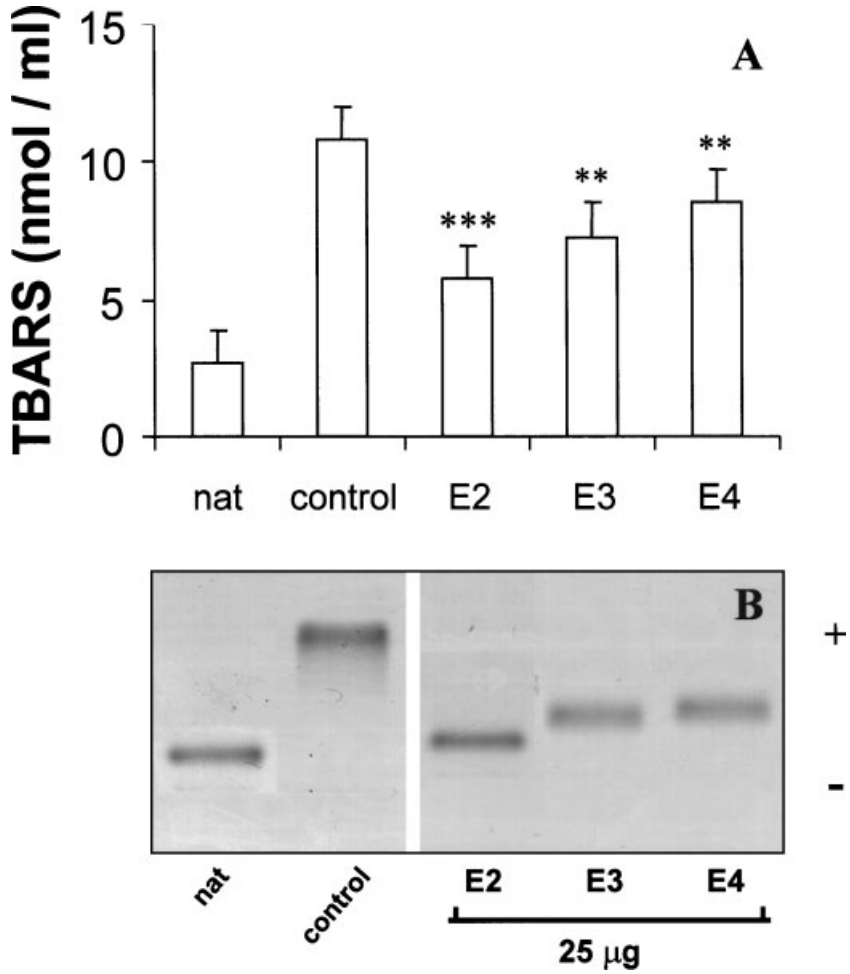

Fig. 3. Effect of 25 μg/ml exogenous recombinant apoE2, E3, and E4 on LDL oxidation by copper. One hundred fifty micrograms of LDL were incubated with 10 μM $CuSO_4$ during 16 h. Oxidation was assessed by measuring the formation of TBARS in the medium (**A**) and the electrophoretic mobility of LDL (**B**). Data are means ± SD of three independent experiments. Statistically significant differences from control (no apoE): $^{**}P < 0.01$ and $^{***}P < 0.001$. Nat: native LDL.

TABLE III. Macrophage Intracellular Content in Cholesterol After Incubation With LDL

Assay conditions	Cell total cholesterol (μg/mg cell protein)
Before incubation with LDL	
ApoE-deficient J774	20.53 ± 0.18*
After incubation with LDL	
ApoE-deficient J774	35.4 ± 0.53**
ApoE2-J774	22.7 ± 2.89
ApoE3-J774	28.2 ± 1.60***
ApoE4-J774	29.6 ± 1.63***

LDL (150 μg/ml) was incubated with apoE-deficient or transfected J774 macrophages during 16 h. Cells were washed, lysed, and intracellular total cholesterol measured. Data are from three separate experiments and are expressed as mean ± SD.
*$P < 0.001$, significantly different from control apoE-deficient cells after incubation with LDL.
**$P < 0.01$, significantly different from apoE-expressing cells.
***$P < 0.01$, significantly different from apoE2-expressing cells.

essentially in relation to Alzheimer's disease [Miyata and Smith, 1996; Smith et al., 1998; Fernandes et al., 1999; Ramassamy et al., 2001]. In the present work, we have investigated these properties in the framework of macrophage-mediated LDL oxidation.

We have shown that secreted apoE isoforms (E2, E3, and E4) are able to protect LDL from oxidation by macrophages. This was demonstrated using different indices of oxidation: PUFA consumption, formation of TBARS, and relative electrophoretic mobility. Measurement of fatty acid consumption (Fig. 1A,B) is a very specific way to evaluate LDL oxidation [Sattler et al., 1991]. Due to its higher number of double-bonds, which renders it more sensitive to oxidation, AA (C20:4) was much more oxidized than LA (C18:2) over 16 h of incubation of LDL with J774 macrophages. Measurement

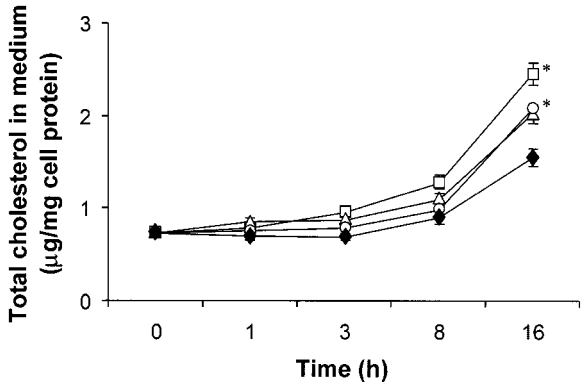

Fig. 4. Cholesterol efflux from macrophages expressing apoE2, E3, or E4. ApoE2-, E3-, or E4-transfected J774 macrophages were incubated with 150 µg LDL over 16 h, washed, and incubated in the efflux medium for a further 3, 8, or 16 h. At the end of these respective periods, total cholesterol was extracted from the medium as described under Material and Methods. Black diamonds: control (no apoE), squares: apoE2, triangles: apoE3, circles: apoE4. Data are means ± SD of three independent experiments. Statistically different from oxidized control: $^*P < 0.05$.

of TBARS (Fig. 1C) is less specific as these substances include all aldehydic breakdown products of oxidized LDL. Nonetheless, this assay confirmed results obtained with the previous fatty acid assay. Finally, aldehydes generated during the oxidative modification of LDL form adducts to the epsilon amino groups of lysine in the apo B moiety of LDL [Steinbrecher, 1987]. This leads to a net increase in negative charge resulting in modified LDL electrophoretic mobility, as observed when comparing oxidized LDL to native LDL (Figs. 2B, 3B). Thus, shift in electrophoretic mobility is a further indication of protein oxidation that is assumed to be the final phase of LDL oxidation. In our study, this assay showed evidence of apoE protection and isoform specificity (Fig. 1D).

In order to further investigate the apparent antioxidant effect of apoE, we demonstrated the ability of exogenous apoE to inhibit free radical-mediated LDL oxidation by demonstrating its capacity to increase the lag time of conjugated diene formation in a cell-free system (Fig. 2 and Table II). Conjugated dienes are intermediate products formed during the peroxidation of PUFAs in LDL phospholipids and the lag time duration of their formation represents the resistance of LDL to oxidation. Our results are consistent with the work of Lin et al. [2002] who reported the contribution of HDL-apolipoproteins to the inhibition of LDL oxidation either in a cell-free system or by apo-E deficient J774 macrophages. However, their study did not allow discrimination between apoE and apoAI in this effect, rather it focused on an exogenous effect, while we were interested in the macrophage ability to protect LDL against oxidative injuries through its own components, such as apoE.

The protective effect of apoE on macrophage-mediated LDL oxidation that we observed was isoform-specific, but surprisingly apoE2 was the least effective isoform. This result was quite unexpected, given that apoE4 has been reported to have less antioxidant potential than apoE2. However, this was demonstrated either indirectly [Ramassamy et al., 1999] or in cell-free oxidation models [Miyata and Smith, 1996]. The generation of highly reactive oxygen species by the cell has been described as the most relevant mechanism for LDL oxidation. In biological systems, the primary free radical is superoxide, produced by the respiratory chain. Superoxide itself is not reactive enough to generate lipid peroxidation but, in association with hydrogen peroxide, it can be converted into the highly reactive hydroxyl radical via the metal-catalyzed Fenton reaction [Westhuyzen, 1997]. Thus metals, namely Fe^{2+} and Cu^{2+}, can be important for the initiation of lipid peroxidation [Aust et al., 1985]. A more direct demonstration of apoE capacity to prevent metal-induced lipoprotein oxidation has been provided by Miyata and Smith [1996], who were the first to consider a possible protective role for apoE against oxidative reactions in the context of Alzheimer's disease. They demonstrated, under in vitro conditions, that apoE was able to (i) quench the enhanced chemiluminescence reaction of a luminol substrate oxidized by H_2O_2 and horseradish peroxidase, (ii) inhibit the oxidation of lipoproteins by copper, and (iii) bind certain metal ions, mainly Cu^{2+} and Fe^{2+}. It was thus concluded that apoE possessed an antioxidant activity likely due to its capacity to chelate metal ions. Furthermore, this activity was shown to be allele-specific (E2 > E3 > E4) [Miyata and Smith, 1996]. How then can we explain our results in macrophages where apoE4 was more efficient than apoE2? In our experiments, the incubation medium (Ham's F10 medium) for macrophage-mediated LDL oxidation contained traces of iron and copper, therefore oxidative processes occurring in this medium could theoretically be initiated by these

ions and eventually be prevented by metal chelation. When LDL was incubated in a cell-free system containing copper in the presence of various concentrations of apoE2, E3, or E4, at a concentration of 25 μg/ml, apoE inhibited LDL oxidation in an isoform-specific way, E2 being the most efficient, thereby confirming the results of Miyata and Smith [1996]. This prompts us to suggest that, in the macrophage-mediated oxidation model, either metal-induced oxidation is a minor event in oxidizing LDL, or apoE does not exert its antioxidant role mainly by inhibiting the metal-mediated oxidative processes.

Nevertheless, in order to further investigate the allele-specificity of apoE in preventing LDL oxidation by macrophages, we assessed its relationship to the intracellular cholesterol content and the ability of apoE to efflux cholesterol in the medium. Accordingly, we found that the intracellular total cholesterol content was significantly lower in apoE2-expressing cells than in those expressing apoE3 or E4 (Table III) and that cells expressing apoE2 effluxed significantly more cholesterol in the medium than cells expressing the two other isoforms (Fig. 4). Considering that cholesterol is a very good substrate for oxidation, the apoE2-mediated larger efflux of cholesterol could account for an increased production of oxidative indices in the medium and, consequently, increased LDL oxidation compared to that observed with apoE3 or E4. This possibility is relevant from a pathophysiological point of view, given that the macrophage environment found at lesion sites is an oxidation-prone area and oxysterols are present in atheromas [Hoppe et al., 1997]. It has to be noted, however, that, as expected from numerous studies [Lin et al., 1999], we also showed that apoE-expressing cells are more efficient at effluxing cholesterol than apoE-deficient cells, whatever the isoform. This observation could argue against the antioxidant capacity of apoE. Alternatively, this also suggests that the preventive effect observed with apoE might result from a balance between pro- and anti-oxidant events and that the apoE trapping of free radicals may be stronger in protecting LDL than the apoE ability to efflux cholesterol in favoring oxidative reactions. In other words, in the macrophage-mediated oxidation model, it is the relative contribution of these two phenomena that will determine the outcome.

In summary, our results have shown that macrophage-secreted apoE contributes to protecting LDL from oxidation in an isoform-specific way, apoE2 being less effective than apoE3 and apoE4, contrary to what we and others have observed in cell-free systems. We believe that our results could at least in part be explained by the increased accumulation of cholesterol in the medium of apoE2-transfected macrophages, a potential source of oxidative indices. This possibility underlines the complexity of the experimental models used, further stressing that secreted apoE in the macrophage-mediated oxidation model and exogenous apoE in metal-induced oxidation may yield different results. However, our results also emphasize the need for careful selection of the apoE isoform to be targeted, especially in the perspective of apoE transgene use. Although we found that apoE4 was more efficient at preventing LDL oxidation than apoE2 in the macrophage-mediated oxidation model, our results and that of others in cell-free systems and, above all, the repeated association of apoE4 with human atherogenesis [Davignon et al., 1999], would suggest that apoE4 is not a suitable isoform to target for therapy. More experiments need to be done in order to determine whether E2 or E3 constitutes a good candidate. For example, it becomes important to know more about the efficiency of removal from the arterial wall of the cholesterol effluxed by apoE from macrophages. If it remains in the macrophage environment, apoE may be considered to participate in pro-atherogenic events such as increasing LDL oxidation indices. However, if cholesterol is rapidly removed from the macrophage environment, then apoE may contribute to slowing down foam cell accumulation and preventing atherogenesis.

In addition, more information about the exact mechanisms involved in in vivo LDL oxidation is needed. In particular, very little is known about the relative contribution of metal-induced oxidation of LDL and cholesterol efflux in atherogenesis. Targeting a specific apoE isoform to the arterial wall in the animal will be necessary to precisely address such questions.

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