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Antioxidant BO-653 and Human Macrophage-Mediated LDL Oxidation

KARIN MÜLLER*, KERI L.H. CARPENTER, MARK A. FREEMAN and MALCOLM J. MITCHINSON

Division of Cellular Pathology, Department of Pathology, University of Cambridge, Tennis Court Road, Cambridge CB2 1QP, UK

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Oxidation of LDL is now widely accepted to be involved in atherogenesis. The aim of this study was to examine the effect of BO-653, a strong radical scavenger and antioxidant, on oxidation of LDL by human macrophages *in vitro*. Fifty μg/ml LDL protein was incubated with macrophages in Ham's F10 medium, supplemented with additional Fe^{2+}, for up to 48 h. Then the medium was analysed by LDL agarose gel electrophoresis, the thiobarbituric acid assay and gas chromatography. In the absence of added exogenous antioxidants, after 24 h LDL oxidation produced 30.48 nmoles MDA equivalents/mg LDL protein and a relative electrophoretic mobility of 4.74. Linoleic acid (18 : 2), arachidonic acid (20 : 4) and cholesterol were depleted and 7β-hydroxycholesterol was generated. BO-653 completely inhibited this cell-mediated oxidation of LDL in concentrations as low as 5 μM, being more effective than either α-tocopherol or probucol, which completely inhibited oxidation at 200 and 80 μM and only partially at 80 and 8 μM, respectively. This inhibition of cell-mediated LDL oxidation was not due to toxicity, as α-tocopherol, probucol and BO-653 were not toxic for the macrophages at the concentrations tested. Eighty μM α-tocopherol, 8 μM probucol and 5 μM BO-653 significantly reduced the toxicity to the oxidising culture caused by LDL oxidation. The results show that in this system BO-653 is a more effective antioxidant than α-tocopherol or probucol.

Keywords: BO-653, low density lipoprotein, oxidation, macrophages (human), antioxidants, atherosclerosis

INTRODUCTION

There is now clear evidence that oxidation of low density lipoprotein (LDL) occurs in human atherosclerotic lesions. The serum of patients with advanced atherosclerosis contains antibodies against oxidised LDL (oxLDL)[1] and oxLDL is identified in human atherosclerotic lesions by antibodies raised against LDL oxidised *in vitro*.[2] Oxidation products of cholesterol, including 7β-hydroxycholesterol, accumulate in advanced human plaques,[3] whereas polyunsaturated fatty acids and α-tocopherol are depleted.[3,4] OxLDL is thought to be involved in atherogenesis in several ways. It is chemotactic for human monocytes *in vitro*,[5] probably contributing to the recruitment of monocyte-macrophages into the intima. The excessive uptake of oxLDL by macrophages seen *in vitro*[6–8] may

*Corresponding author. Tel.: ++44 1223 333460. Fax: ++44 1223 333872. E-mail: km3@mole.bio.cam.ac.uk.

cause foam cell formation *in vivo*. Further, oxLDL is toxic to various cell types *in vitro*,[9–11] including monocyte-macrophages, and foam cell death contributes to the enlargement of the lipid core during lesion progression.[12]

The effectiveness of antioxidants, such as α-tocopherol, β-carotene and probucol, in the prevention of atherosclerosis has been addressed in numerous studies and some have given promising results.[13] However, new antioxidants are being investigated in the search for therapeutically useful compounds. 2,3-Dihydro-5-hydroxy-2,2-dipentyl-4,6-di-*tert*-butylbenzofuran (BO-653) is a novel, phenolic radical scavenger chemically designed for high antioxidant activity.[14] In a recent study using electron spin resonance, its effect on lipid peroxidation *in vitro* was compared with that of α-tocopherol.[14] The reactivity of BO-653 towards peroxyl radicals was smaller than that of α-tocopherol. However, the radical derived from BO-653 was more stable than that derived from α-tocopherol, probably due to the introduction of *tert*-butyl groups into the molecule. This substitution also increased the lipophilicity of BO-653 and may be responsible for its greater potency against lipid peroxidation in phosphatidylcholine liposomal membranes as compared to α-tocopherol.[14] Furthermore, BO-653 inhibits the oxidation of LDL induced by azo radical initiators *in vitro*.[15] These data indicated that BO-653 may act as a strong antioxidant *in vitro* and *in vivo*. The aim of this study was to compare the potency of BO-653, in inhibiting the oxidation of LDL by human macrophages, with those of α-tocopherol and probucol.

MATERIALS AND METHODS

Chemicals

BO-653 was supplied by Chugai Pharmaceutical Company, Gotemba, Shizuoka, Japan. α-Tocopherol (T-3251) and probucol (P-9672) were purchased from Sigma-Aldrich (Poole, Dorset, UK). Antioxidants were kept under argon at −70°C. Stock solutions were prepared in pure ethanol at 50 mM for α-tocopherol and 20 mM for probucol and BO-653 and kept under argon at −20°C in the dark. Organic solvents were purchased from Fisher (Leicester, UK) or BDH (Poole, Dorset, UK) and were of analytical grade or better. Chemicals and standards were from Sigma-Aldrich (Poole, Dorset, UK) and were of the highest purity available. Silylating reagent (*N*, *O*-bis(trimethylsilyl)-trifluoroacetamide plus 1% trimethylchlorosilane) was from Pierce and Warriner UK, Chester, UK.

Isolation of LDL

LDL samples were isolated from pooled human plasma of 5 or more healthy volunteers by ultracentrifugation in potassium bromide gradients according to Havel *et al.*[16] in the presence of 1 mM EDTA. Native LDL was dialysed extensively against 10 mM Tris/HCl buffer pH 7.4 containing 0.15 M NaCl and 1 mM EDTA, filter-sterilized (0.45 μM filters) and stored at 4°C for a maximum of 4 weeks. Protein concentrations of LDL were measured according to Lowry and colleagues[17] using bovine serum albumin (BSA) as standard. Prior to addition to the cells, LDL was dialysed for 24 h against phosphate-buffered saline (PBS) to remove EDTA and filter-sterilized (0.45 μM filter).

Cell Culture

Mature human macrophages (Mϕ) were obtained by prolonged *in vitro* culture of human monocytes[18,19] isolated from HIV-screened human buffy coat (National Blood Service, Brentwood, UK). Thirty ml of buffy coat was layered on to 15 ml of LymphoPrep (Nycomed Pharma AS, Oslo, Norway) and after centrifugation at room temperature for 30 min at 600× *g* the opaque interphase of mononuclear cells was removed and washed twice with PBS containing 4 mg/ml BSA to remove platelets. Then, monocytes were

prepared by an additional centrifugation step in a Percoll gradient.[20] Mononuclear cells were resuspended in 4 ml of PBS and mixed with 8 ml of Percoll: Hank's Balanced Salt Solution (10× concentrate) (6 : 1; at pH 7.0). After centrifugation at room temperature for 30 min at 400 × g the monocytes were collected from the top of the gradient, washed twice in PBS/BSA and seeded in 24-well and 48-well tissue culture plates (Becton Dickinson, Franklin Lakes, New Jersey, USA) at a density of 5×10^5 and 2.5×10^5 cells/well, respectively. After incubation at 37°C for 1 h, any remaining non-adherent cells were removed by washing three times with PBS. Adherent monocytes were cultured in serum-free medium (SFM) macrophage-SFM (Gibco BRL, Paisley, Scotland, UK) for up to 21 days, renewing half of the culture medium in each well twice a week so as to minimise disturbance of cells. The purity of monocyte-macrophage preparations was examined after staining with the Simultest LeucoGATE kit (Becton Dickinson, San José, CA, USA) using LYSYS II software on a FACScan flow cytometer (Becton Dickinson). On average monocyte-macrophage purity was 90%.

Cell-mediated Oxidation of LDL

Cell oxidation experiments were performed with Mϕ after 14–21 days in culture in Ham's F10 medium (ICN Biomedicals Inc., Costa Mesa, CA, USA) supplemented with 2 mM L-glutamine and with 7 μM $FeSO_4$ ('oxidation medium') thus bringing the total Fe^{2+} concentration to 10 μM.[10] The concentration of phenol red was 1.2 mg/l, the copper concentration was 0.01 μM as stated by the manufacturer. Cells were washed twice with PBS to remove the culture medium and native LDL was added at 50 μg/ml LDL protein (1 ml per well). Antioxidants were added simultaneously at the indicated concentrations. After incubation for up to 48 h, the supernatants were removed and spun at 2000× *g* for 5 min to remove any detached cells. The supernatant was transferred to fresh tubes and 20 μM BHT and 5 μM EDTA were added to prevent further oxidation. The degree of LDL oxidation was measured immediately by agarose gel electrophoresis and by the thiobarbituric acid assay. For GC analysis the supernatant of two identical wells was combined (2 ml) and stored at −70°C until extraction. As a measure of non-biological oxidation, identical LDL samples were incubated under the same conditions in the absence of cells (NC-control).

Thiobarbituric Acid Assay

The formation of aldehydic peroxidation products in LDL was estimated by the thiobarbituric acid (TBA) assay using 1,1,3,3-tetramethoxypropane as standard[21] and Ham's F10 medium for all dilutions. Five hundred μl of culture supernatant was mixed with 250 μl of thiobarbituric acid (1.34% (w/v)) and 250 μl of trichloroacetic acid (40% (w/v)). After incubation at 90°C for 30 min, samples were spun at 15,000× *g* for 10 min and the absorbance of the supernatants was read at 532 nm. The degree of oxidation is expressed as nmoles malondialdehyde (MDA) equivalents/mg LDL protein.

Agarose Gel Electrophoresis

The electrophoretic mobility of LDL was examined by agarose gel electrophoresis on Paragon 'LIPO' gels (Beckman, Brea, CA, USA). Five μl of sample was applied to each lane and, after running for 30 min at 100 V, the gels were fixed for 5 min in ethanol : deionised water : glacial acetic acid (60 : 30 : 10, v/v), dried and stained with Paragon LIPOstain (Sudan Black B, Beckman, Brea, CA, USA). The relative electrophoretic mobility (REM) was calculated as the migration of sample in mm divided by the migration of native LDL in mm.

Extraction and Workup of Lipids

Lipids were extracted from the medium and processed for GC analysis as described previously.[22] The procedure comprised adding

internal standards (n-heptadecanoic acid, 5α-cholestane and coprostane), Bligh and Dyer extraction (with sonication), sodium borohydride reduction, saponification and derivatisation to methyl esters and trimethylsilyl ethers. Processed samples were stored at −20°C under argon until GC analysis. Care was taken to minimise exposure of samples to air, and sodium borohydride reduction, saponification, derivatisation and storage of samples were all under nitrogen or argon.

Analysis by Gas Chromatography

After the above work-up, GC analysis[22] of the lipid extracts was performed using a 30 m DB-1 fused silica capillary column (0.32 mm i.d., film thickness 0.1 μM) (J&W. Scientific, Folsom, CA, USA) on a Carlo Erba Vega 6130 gas chromatograph equipped with an automated split-splitless injector operated in the splitless mode, and a flame ionisation detector. Samples were injected as hexane solutions. The column oven was temperature programmed at 50–120°C at 10°C/min, 120–200°C at 4°C/min, 200–280°C at 3°C/min, and held at 280°C for 10 min. Quantitation was by peak areas, measured electronically using a Carlo Erba DP800 integrator, relative to the internal standards.

^{3}H-Adenine Assay

Plasma membrane integrity of Mϕ was measured in 48-well plates using a tritiated adenine assay based on that of Andreoli and colleagues.[23] After washing twice with PBS, cells were loaded with 0.25 μCi (9.25 kBq) [8-^{3}H]adenine (Amersham Radiochemicals Ltd., Aylesbury, Bucks, UK) for 1 h at 37°C. Then, non-incorporated tritiated adenine was removed by washing the cells three times with PBS. Subsequently, cells were incubated with test samples as indicated. Supernatants were removed, spun for 5 min at 1000× *g* to remove any detached cells and the supernatant transferred to fresh tubes. Cells remaining in the wells were lysed in 500 μl of Triton X-100 (1% (v/v)) and combined with the cell pellet of the centrifugation. Two hundred μl each of supernatant and lysate were mixed with 3 ml of OptiPhase 'Hi-Safe' scintillant (Fisons Chemicals, Loughborough, Leics., UK) and radioactivity was measured using a Packard Tricarb 1600 TR liquid scintillation analyser. The percentage of radioactivity leakage was calculated as: 100× dpm supernatant/(dpm supernatant + cell lysate).

Statistics

The significance of results was calculated using the unpaired Student's *t*-test on Microsoft Excel 7.0. Graphs were prepared using FigP software.

RESULTS

BO-653 and Cell Viability

We examined the effect of BO-653, α-tocopherol and probucol on various parameters of Mϕ viability *in vitro* using the same culture medium as for the oxidation experiments but in the absence of LDL. BO-653 was tested at 0.5 and 20 μM, α-tocopherol at 80 and 200 μM and probucol at 8 and 80 μM.

The result of the tritiated adenine assay, which monitors plasma membrane integrity, is shown in Figure 1. BO-653 did not significantly increase the radioactivity leakage over 48 h compared to the medium control (Figure 1A). Similarly, α-tocopherol and probucol were not significantly toxic for human Mϕ over 48 h (Figure 1B and C). The effect of antioxidants on the lysosomal activity of human Mϕ was examined using the neutral red assay. Neutral red is only accumulated in lysosomes of living cells and a decrease in lysosomal activity is detected as a decrease in absorbance at 540 nm. Neither BO-653, nor α-tocopherol or probucol significantly altered lysosomal activity of Mϕ over 48 h compared to the medium control (data not shown). The MTT assay was used to examine the effect of antioxidants on the activity of dehydrogenase enzymes within mitochondria

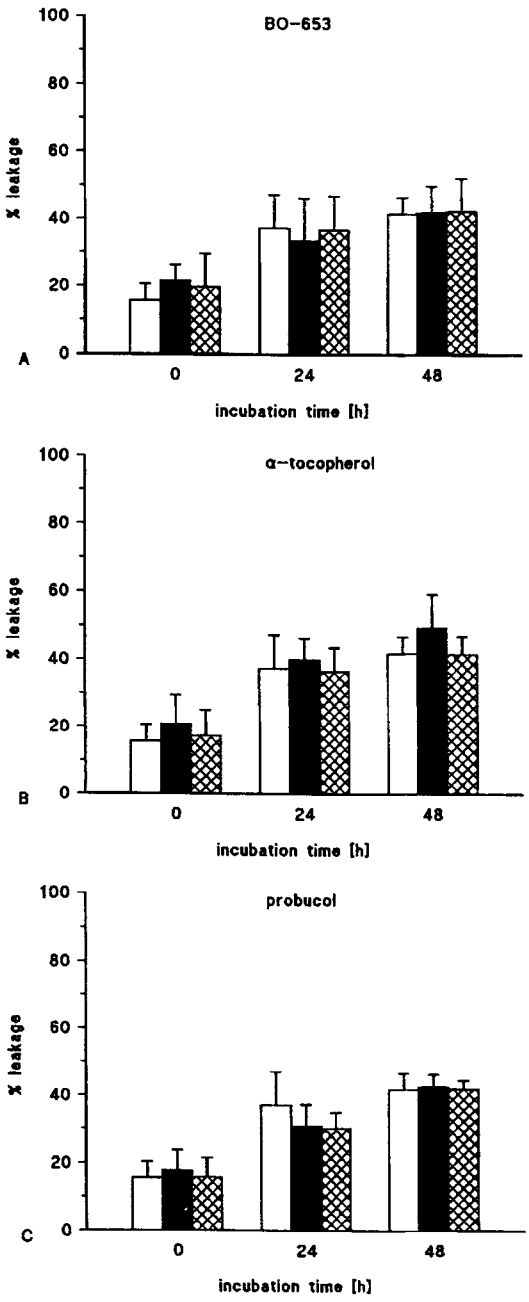

FIGURE 1 Tritiated adenine assay. Human Mϕ were loaded with 0.5 μCi/ml tritiated adenine for 1 h. Subsequently, the cells were incubated for up to 48 h with medium only (open bars) or with (A) 20 μM (solid bars) and 0.1 μM BO-653 (cross-hatched bars), (B) 200 μM (solid bars) and 80 μM (cross-hatched bars) α-tocopherol or with (C) 80 μM (solid bars) and 8 μM (cross-hatched bars) probucol. Results are given in percentage radioactivity leakage and represent the mean ± standard deviation of two separate experiments performed in duplicate.

and the endoplasmic reticulum of Mϕ. The yellow MTT dye is converted to a blue formazan dye by living cells only, and a decrease in enzymatic activity is measured as a decrease in absorbance at 570 nm. BO-653 and α-tocopherol did not significantly change the absorbance at 570 nm compared to the medium control over 48 h. Probucol at 80 μM but not at 8 μM led to an increase in absorbance after 48 h compared to the medium control (data not shown).

Taken together, neither BO-653 nor α-tocopherol or probucol were toxic to human Mϕ over 48 h at the concentrations used in our experiments.

Influence of BO-653 on Cell-mediated Oxidation

In the present study, mature human Mϕ were incubated with 50 μg/ml LDL protein in the absence or presence of antioxidants for up to 48 h. Ham's F10 was used as oxidation medium and supplemented with an additional 7 μM Fe^{2+} in order to avoid limitation of cell-mediated oxidation by lack of transition metals.[10] The degree of LDL oxidation was measured using the TBA assay, agarose gel electrophoresis and analysis by GC.

Lipid Peroxidation

In the absence of cells an average increase in LDL oxidation from 4.15 nmoles MDA/mg LDL protein at 0 h to 11.16 nmoles MDA/mg LDL protein at 24 h was measured due to non-biological oxidation. No significant effect of the antioxidants on this non-biological oxidation was apparent (data not shown). In the presence of Mϕ, LDL was oxidised to 30.48 nmoles MDA/mg LDL protein within 24 h (Figure 2). Two hundred μM α-tocopherol and 80 μM probucol completely inhibited cell-mediated LDL oxidation, whereas 80 μM α-tocopherol and 8 μM probucol resulted only in a partial inhibition. At concentrations of 5 μM and above BO-653 completely inhibited LDL oxidation, whereas concentrations of BO-653 of 1 μM and below were ineffective.

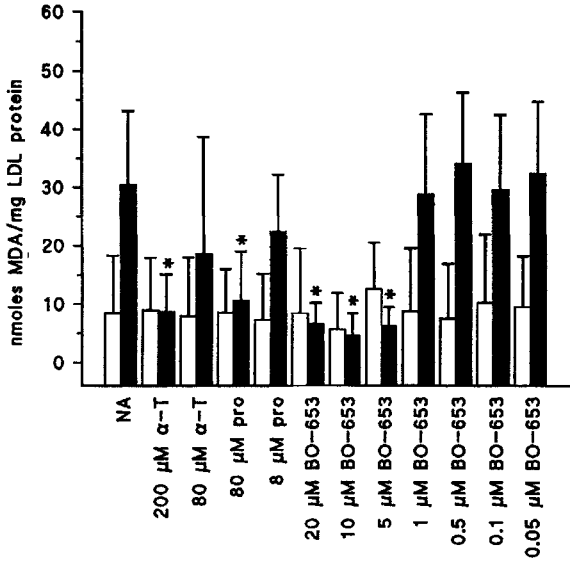

FIGURE 2 Lipid peroxidation of LDL. Fifty µg/ml LDL protein was incubated with mature human macrophages for 0 h (open bars) and 24 h (solid bars) in the absence (NA) or presence of antioxidants. Results are the mean ± standard deviation of three separate experiments (* = $p \leq 0.05$).

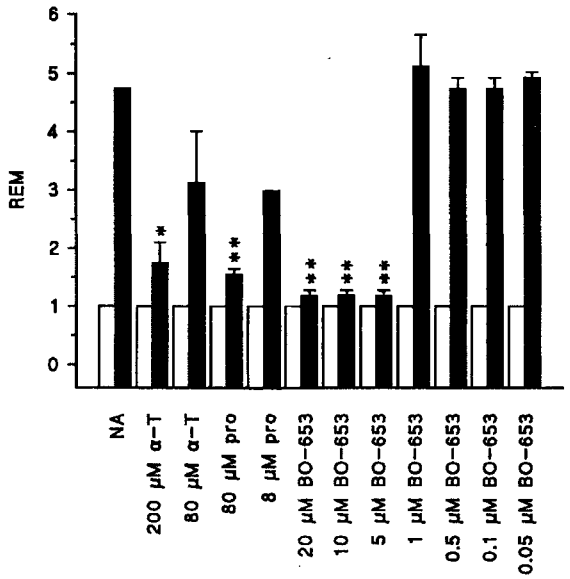

FIGURE 3 Relative electrophoretic mobility (REM) of LDL. Fifty µg/ml LDL protein was incubated with mature human Mϕ for 0 h (open bars) and 24 h (solid bars) in the absence (NA) or presence of antioxidants. Results are the mean ± standard deviation of two separate experiments (* $p \leq 0.05$, ** $p \leq 0.001$).

Electrophoretic Mobility

Incubation of 50 µg/ml LDL protein in the absence of cells for 24 h moderately increased the electrophoretic mobility of LDL (average REM 1.5) due to non-biological oxidation, on which the antioxidants had no significant effect (data not shown). In the presence of Mϕ, native LDL was strongly oxidised with an increase in REM to 4.75 after 24 h (Figure 3). Two hundred µM α-tocopherol and 80 µM probucol inhibited the increase in electrophoretic mobility of cell-oxidised LDL almost completely, whereas 80 µM α-tocopherol and 8 µM probucol effected only a partial inhibition (REM of 3.13 and 3, respectively). BO-653 completely inhibited cell-mediated oxidation of LDL down to a concentration of 5 µM, whereas concentrations of 1 µM and below were ineffective.

Gas Chromatography

The effect of BO-653 on the content of lipids and oxidised lipids in medium during incubation of LDL with Mϕ was examined by gas chromatography (Figure 4). The concentrations of individual components given below are the sum of their free and esterified forms in LDL and no differentiation of the degree of esterification was performed. The results for the Mϕ cultures are expressed as the percentage of the 0 h-time points (100%) or in µg/mg LDL protein. Apart from arachidonic acid (20 : 4), there was little oxidation apparent in the absence of cells (NC-control), with or without antioxidants (data not shown).

Stearic Acid and Oleic Acid

The concentrations of stearic acid (18 : 0) and oleic acid (18 : 1) in LDL at the 0 h-time point were 44.0 µg/mg LDL protein and 133.4 µg/mg LDL protein, respectively. Their levels remained unchanged during the oxidation of LDL by Mϕ for 48 h (data not shown) and the presence of antioxidants had little effect on their concentrations.

Linoleic Acid and Arachidonic Acid

At the 0 h-time point the concentrations of linoleic acid (18 : 2) and arachidonic acid (20 : 4) in LDL were 256.6 µg/mg LDL protein and 41.35 µg/mg LDL protein, respectively. After 48 h of incubation with Mϕ the levels were decreased to 4.1% and to nearly undetectable levels, respectively (Figure 4A) as compared to 70.0% and 56.6% in the NC-controls. The cell-mediated depletion of 18 : 2 and 20 : 4 was almost entirely inhibited

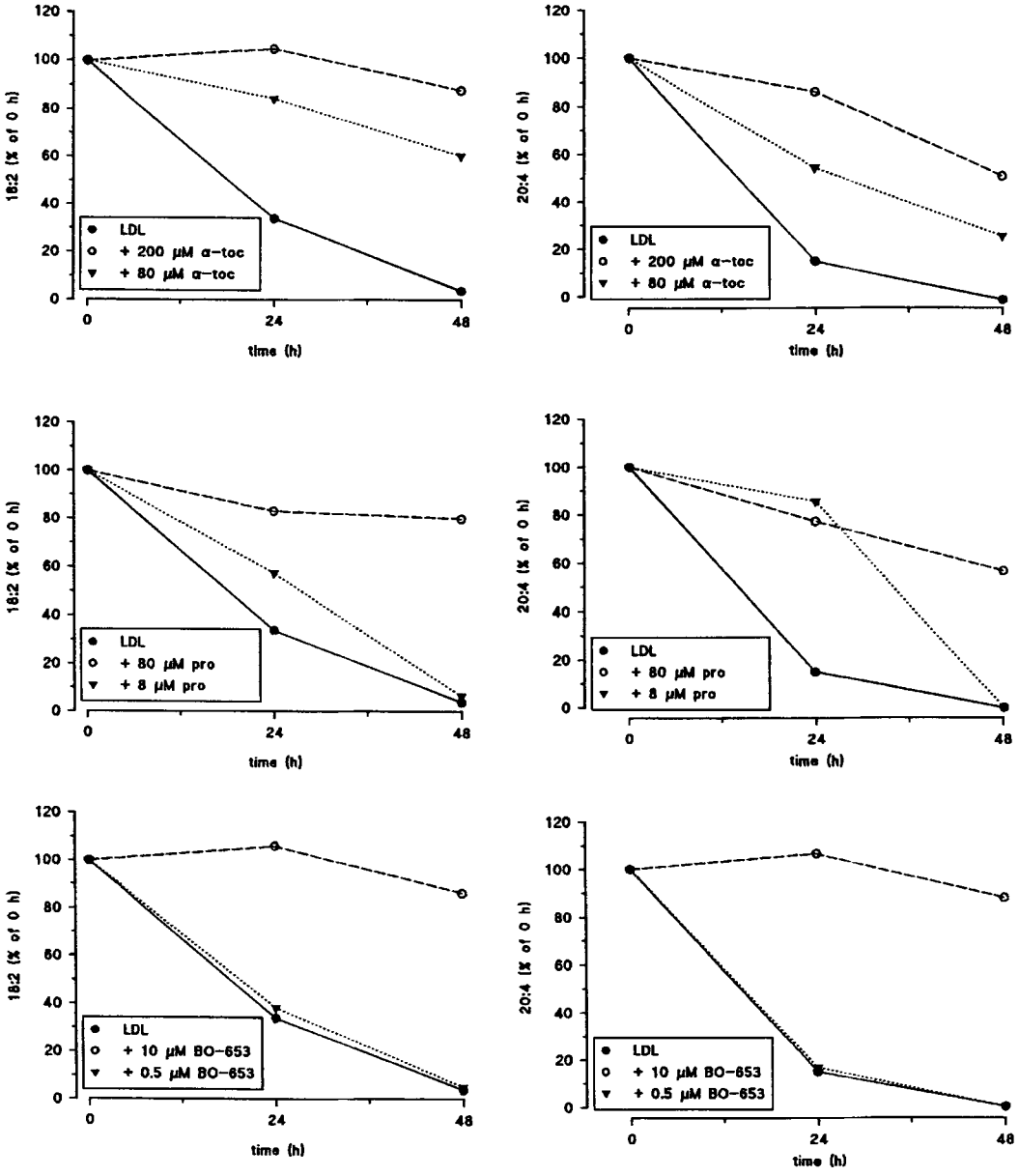

FIGURE 4(a)

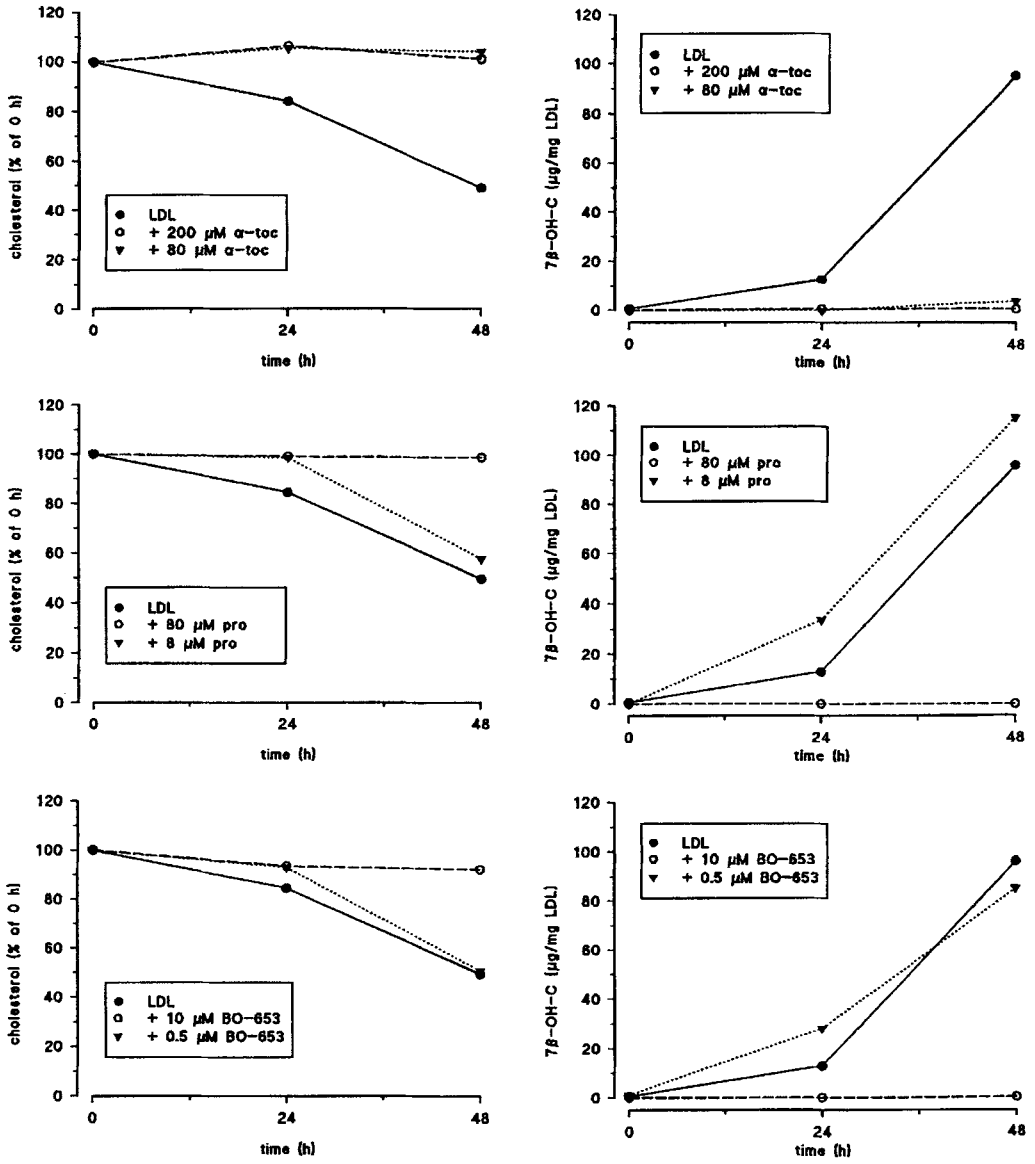

FIGURE 4(b)

FIGURE 4 GC-analysis of Mϕ-oxidised LDL. Fifty µg/ml LDL protein was incubated with Mϕ over 48 h in the absence or presence of antioxidants. The medium was extracted and the concentrations of (A) linoleic acid (18:2) and arachidonic acid (20:4) and (B) cholesterol and 7β-hydroxycholesterol were measured by GC. With the exception of 7β-OH-C, which is in µg/mg LDL protein, the results are given as percentage based on the 0 h-time point (100%). The values represent a single experiment.

by 200 µM α-tocopherol and 80 µM probucol, partially by 80 µM α-tocopherol and not at all by 8 µM probucol and 0.5 µM BO-653. 10 µM BO-653 almost entirely inhibited the depletion of 18:2 (Figure 4A) and in the case of 20:4 even appeared to inhibit almost completely the depletion due to non-biological oxidation (data not shown).

Cholesterol

The concentration of cholesterol in LDL at 0 h was 820.4 μg/mg LDL protein. During incubation with Mϕ for 48 h cholesterol was depleted to 49.3% (Figure 4B) as compared to 87.4% in the NC-control. The presence of 80 and 200 μM α-tocopherol, 80 μM α-probucol and 10 μM BO-653 completely inhibited cholesterol depletion. In the presence of 8 μM probucol and 0.5 μM BO-653 cholesterol was depleted to 57.4, and 50.75%, respectively.

7β-Hydroxycholesterol

During the incubation of LDL with Mϕ for 48 h the level of 7β-hydroxycholesterol (7β-OH-C) rose from nearly undetectable levels to 96.05 μg/mg LDL protein (Figure 4B). This corresponds to 12.65% of the original cholesterol. Only 7.3 μg/mg LDL protein 7β-OH-C were generated in the NC-control after 48 h. Eighty μM and 200 μM α-tocopherol, 80 μM probucol and 10 μM BO-653 completely inhibited the formation of 7β-OH-C, whereas 8 μM probucol and 0.5 μM BO-653 were ineffective (Figure 4B).

Toxicity of Cell-mediated Oxidation

We compared the effect of antioxidants on the toxicity of LDL oxidation to the Mϕ, using the tritiated adenine assay (Figure 5). After 48 h, the oxidation of LDL by Mϕ led to significantly increased release of radioactivity (70.05%) compared to the medium control which did not contain LDL (40.82%). This toxicity was significantly inhibited in the presence of 80 and 200 μM α-tocopherol, 8 and 80 μM probucol and of 5–20 μM BO-653. Concentrations of BO-653 of 1 μM and below did not inhibit this toxicity.

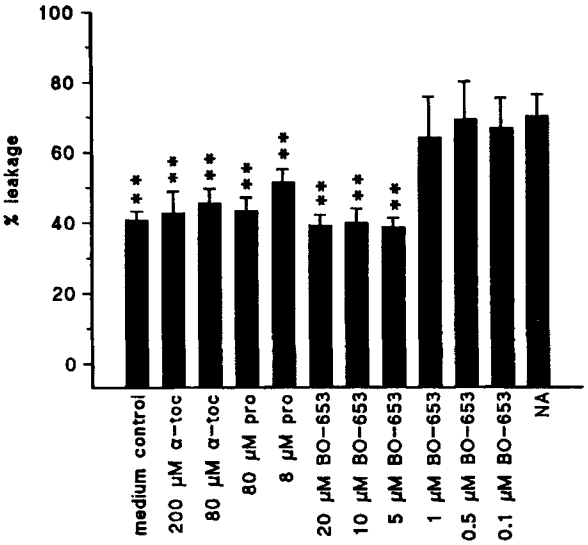

FIGURE 5 Toxicity of cell-mediated oxidation. Mature human Mϕ were pre-loaded with 0.5 μCi/ml tritiated adenine for 1 h and subsequently incubated for 48 h with medium alone (medium control) or with 50 μg/ml native LDL in the absence (NA) or the presence of antioxidants. Then, the radioactivity leakage was measured by liquid scintillation counting. Results are given as percentage radioactivity leakage and represent the mean ± standard deviation of two separate experiments performed in quadruplicate ($^{*}p \leq 0.05$, $^{**}p \leq 0.001$).

DISCUSSION

α-Tocopherol and probucol have previously been shown to inhibit the oxidation of LDL by Cu^{2+} [9,24–26] and also cell-mediated LDL oxidation[10,24,26] probucol usually being the more potent.[9,10] The results of this study show that BO-653 is more potent at inhibiting macrophage-mediated oxidation of LDL *in vitro* than either α-tocopherol or probucol. Five μM BO-653 completely inhibited the increase in TBARS and relative electrophoretic mobility, whereas only partial inhibition was obtained with 80 μM α-tocopherol and 8 μM probucol. Oxidation of LDL by Cu^{2+} or cells leads to a decrease in polyunsaturated fatty acids and cholesterol in LDL and to the generation of cholesterol oxidation products, such as 7β-OH-C.[10,27] Five μM BO-653 completely inhibited the decrease in linoleic acid (18:2) and arachidonic acid (20:4), whereas partial inhibition was obtained with 80 μM α-tocopherol and 8 μM probucol. Depletion of cholesterol and formation of 7β-OH-C were

completely inhibited by 5 μM BO-653, 80 μM α-tocopherol and 80 μM probucol. The inhibition of cell-mediated LDL oxidation was not due to toxicity, as none of the antioxidants was toxic to the cells at the concentrations used in this study. α-Tocopherol and probucol were previously shown to inhibit non-biological oxidation of LDL.[10] In our study an apparently slight inhibition of non-biological LDL oxidation by the antioxidants was observed, which was not statistically significant.

LDL oxidised by cells or copper is toxic to a variety of cells,[11,28] including monocyte-macrophages.[9,10] α-Tocopherol and probucol have been shown to protect human monocytes from the toxicity of Cu^{2+}-oxidised LDL[9] and from the toxicity of LDL oxidation to the oxidising culture.[10] In our study, 5 μM BO-653 completely inhibited the toxicity of LDL oxidation to the oxidising macrophages. Eighty μM α-tocopherol and 8 μM probucol also significantly reduced the toxicity, although they only effected a partial inhibition of lipid peroxidation. This may indicate that even a partial inhibition of lipid peroxidation in LDL is sufficient to lower cytotoxicity. Alternatively, the protection from toxicity may be due to the inhibition of cholesterol depletion and the concomitant decrease in 7β-OH-C formation. Eighty μM α-tocopherol, but not 8 μM probucol, completely inhibited the depletion of cholesterol and the formation of 7β-OH-C. The present study includes a sodium borohydride reduction step in sample processing, which reduces any hydroperoxides, aldehydes or ketones present to alcohols. Thus the 7β-OH-C detected could originate at least partially from 7β-hydroperoxycholesterol or from 7-ketocholesterol. In a previous study on LDL oxidation by mouse peritoneal macrophages (MPMs), approximately 22% of the measured 7β-OH-C was derived from 7-ketocholesterol.[27] 7α-Hydroxycholesterol has been detected in LDL oxidised by MPMs or copper at about one third of the level of 7β-OH-C[27] and at one fifth of 7β-OH-C in this study. However, detailed quantitation of 7α-hydroxycholesterol was not attempted in both studies, due to its close proximity to the large cholesterol peak. All these oxysterols are toxic to cells *in vitro*,[29,30] and may account at least in part for the toxicity observed in our cultures.

There are several possible explanations for the stronger activity of BO-653 in inhibiting cell-mediated LDL oxidation as compared to α-tocopherol. Unlike α-tocopherol, BO-653 possesses a vacant position on the aromatic ring, so that aromatic substitution, for example by reactive nitrogen oxide species (NO_x) or other electrophiles, should be facilitated.[14] This would be analogous to the known NO_2-trapping ability of γ-tocopherol,[31] which also has a vacant position on its aromatic ring. The role of reactive nitrogen oxide species in macrophage-mediated LDL oxidation is incompletely understood. However, it is well established that peroxynitrite ($ONOO^-$), a powerful oxidant and nitrating species, is formed from the reaction of nitric oxide ($^{\bullet}NO$) with superoxide radical anion ($O_2^{\bullet -}$), two radical species generated simultaneously by activated phagocytes.[31] Peroxynitrite is a potent agent of LDL oxidation *in vitro*, promoting peroxidation of the lipid moiety of LDL as well as reacting with the tyrosine residues of apo B-100.[32] Elevated levels of 3-nitrotyrosine were found in LDL isolated from human atherosclerotic intima.[33] Besides NO_x, other electrophiles which might theoretically be trapped by the vacant position of the aromatic ring of BO-653 include 4-hydroxynonenal and other 4-hydroxy-α,β-unsaturated aldehydes, which are generated during LDL oxidation, and which are toxic.[34] In addition, the vacant position on the aromatic ring of BO-653 might also facilitate the adduction of peroxyl radicals,[14] which are generated during LDL oxidation.[35] ESR studies have shown that the phenoxyl radical derived from BO-653 is more stable than α-tocopheroxyl radicals, and is capable of reducing the latter to regenerate α-tocopherol.[14] This sparing effect might mean that BO-653 could work synergistically with endogenous α-tocopherol in LDL.

The antioxidant potency of BO-653 may not only be determined by its reactivity towards radicals, but also by its location and mobility within LDL. The incorporation of antioxidants into LDL was not assessed in this study. However, unlike α-tocopherol,[36] the efficacy of BO-653 for radical scavenging was shown not to decrease when the radical moved deeper into the LDL particle.[15] This may be because BO-653 is more lipophilic and possesses a shorter side chain than α-tocopherol. There is evidence that LDL oxidation proceeds faster in the core than in the shell[37] and the stronger antioxidant effect of BO-653 compared to α-tocopherol might therefore be due to its location within LDL. It may be located within the core of the particle rather than the surface and may be able to move more freely.[15]

The usefulness of antioxidants in the prevention of atherosclerosis, however, may not depend only on their antioxidant activity. Probucol inhibits atherosclerotic lesion progression in Watanabe Heritable Hyperlipidemic rabbits,[38] whereas both positive and negative results have been obtained in rabbits on a high-cholesterol diet.[39,40] Probucol supplementation also failed to protect against progression of femoral atherosclerosis in humans.[41] The lack of protection against atherosclerosis by probucol in some cases despite its high antioxidant activity *in vitro* has been attributed to its property to lower the plasma concentrations both of high density lipoprotein[42] and of diet-derived antioxidants, such as α-tocopherol and carotenoids.[43] α-Tocopherol has been shown to inhibit atherosclerotic lesion formation in rabbits on a high-cholesterol diet[40] and to reduce the risk of non-fatal myocardial infarction in humans.[44] However, apart from its antioxidant activity, α-tocopherol has other biological effects.[45] For instance, it inhibits the proliferation of smooth muscle cells *in vitro*,[46] the release of inflammatory cytokines, such as IL-1β, by macrophages,[47] and platelet aggregation.[48] The beneficial effect of α-tocopherol on atherosclerosis may be the result of its antioxidant activity alone or in combination with its other biological properties. The present study has shown that BO-653 has a higher potency *in vitro* to inhibit macrophage-mediated oxidation of LDL than α-tocopherol or probucol. More studies investigating the wider biological activities of BO-653 are needed in order to establish whether it might represent a useful compound for the prevention of complications of atherosclerosis *in vivo*.

Notes Added in Proof

During the preparation of this manuscript a study was published which shows that BO-653 is protective against experimental atherosclerosis.[49] This raises the possibility that it might be effective in humans.

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