5-Lipoxygenase is not essential in macrophage-mediated oxidation of low-density lipoprotein

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The concentration-dependent effects of a series of lipoxygenase inhibitors and antioxidants on the macrophage-mediated oxidative modification of low-density lipoprotein (LDL) were measured. Their influence on macrophage 5-lipoxygenase pathway activity was also studied over the same concentration range. No correlation between inhibition of 5-lipoxygenase and of macrophage-mediated oxidation of LDL was observed. The capacity of the compounds to prevent cell-mediated modification of LDL could be explained in terms of their activity as either aqueous- or lipid-peroxyl radical scavengers. Two potent 5-lipoxygenase inhibitors (MK 886 and Revlon 5901), which had no radical-scavenging properties, were unable to block LDL modification. It is concluded that 5-lipoxygenase is not essential for LDL oxidation by macrophages.

INTRODUCTION

The modification of human low-density lipoprotein (LDL) by macrophages has been demonstrated previously [1,2]. This altered LDL has a high affinity for cell-surface receptor(s) on several cell types and is endocytosed by macrophages 10–20 times more rapidly than is native LDL. Cell-modified LDL shows some receptor specificity in common with acetylated LDL [3]. It has been suggested that this cell-mediated alteration to LDL may occur *in vivo* and contribute to foam cell generation in the developing atherosclerotic lesion.

The process of modification involves consumption of antioxidants and the oxidation of the LDL particle [4]. The cellular mechanisms responsible for macrophage-mediated oxidation of LDL are not understood, though it is generally believed that the cells initiate peroxidation of LDL lipids, which subsequently derivatize the apolipoprotein B molecule, altering the affinity of the lipoprotein for several cell-surface receptors [5].

In the present study we have addressed the mechanism of this cell-mediated peroxidative reaction. Of particular interest is the hypothesis that cellular lipoxygenase activity may initiate oxidation in the LDL particle. The reaction catalysed by lipoxygenases involves a radical intermediate and leads to the formation of lipid hydroperoxides. Lipoxygenases may promote LDL oxidation via cellular lipid metabolism, either through the release from the enzyme of a peroxyl radical [6] or indirectly by the oxidation of cell membrane lipids. LDL lipids may also be substrates for cellular lipoxygenases [7]. It has previously been reported that purified soybean lipoxygenase can produce some of the alterations to LDL which are observed during cell-mediated modification [8]. Once LDL lipid peroxidation is initiated, it is propagated by peroxyl radicals within the LDL particle and may be inhibited at this stage by chain-breaking peroxyl radical scavengers such as vitamin E. The hypothesis requires that both lipoxygenase inhibitors and chain-breaking peroxyl radical scavengers should inhibit cell-mediated modification of LDL. There are several reports of the inhibitory activity of antioxidants such as butylated hydroxytoluene (BHT) and α -tocopherol [9,10], which support the latter requirement. Some lipoxygenase

inhibitors have also been shown to block both endothelial and macrophage-mediated oxidative modification of LDL [11,12]. However, many lipoxygenase inhibitors are also peroxyl radical scavengers, so that these compounds must be carefully characterized in testing the hypothesis. To do this, in the present study we have examined the activity of a range of lipoxygenase inhibitors as both inhibitors of macrophage-mediated LDL modification and as peroxyl radical scavengers. To study the latter, we have used a range of models of LDL oxidation *in vitro*. The agents tested included compounds with well-established antioxidant activity (BHT, Ionox-220, Probucol) and lipoxygenase inhibitory activity [BW755C, BWA4C, Revlon 5901, MK 886, nordihydroguaiaretic acid (NDGA) and 5,8,11,14-eicosatetraynoic acid (ETYA)]. We have also studied the activities of lipoxygenase pathways in macrophages and directly measured the inhibitory activity of the agents in this system.

EXPERIMENTAL

Materials

Na¹²⁵I (14 mCi/ μ g) and leukotriene B₄ (LTB₄) ³H radioimmunoassay kit were from Amersham. Ham's F-10 medium (cat. no. 320-1550AJ) and Dulbecco's minimal essential medium (DMEM; cat. no. 320-1885AJ) were from Gibco. Other tissue culture sera and reagents were from Gibco or Flow Laboratories. NDGA and BHT were from Sigma; BWA4C [*N*-(3-phenoxybenzyl)acetohydroxamic acid], BW755C (3-amino-1-[*m*-trifluoromethylphenyl]-2-pyrazoline), Probucol, MK 886 (3-[1-(4-chlorobenzyl)-3-*t*-butylthio-5-isopropylindol-2-yl]-2,2-dimethylpropanoic acid) and Revlon 5901 (2-[3-(1-hydroxyhexyl)-phenoxyethyl]quinoline) were synthesized at the Department of Medicinal Chemistry, Wellcome Research Laboratories (Beckenham, Kent, U.K.). ETYA was from Calbiochem Australia. Chelex-100 was from Bio-Rad. All other chemicals were of analytical grade and were from Merck, Sigma or BDH.

LDL preparation

Human LDL (*d* 1.019–1.050) was isolated from fresh plasma, and collected in EDTA, aprotinin and soybean trypsin inhibitor

Abbreviations used: AAPH, 2,2'-azobis(2-amidinopropane) dihydrochloride; BHT, butylated hydroxytoluene; DMEM, Dulbecco's minimal essential medium; DMSO, dimethyl sulphoxide; ETYA, 5,8,11,14-eicosatetraynoic acid; IC₅₀, concn. resulting in 50% inhibition; LTB₄, leukotriene B₄; LDL, low-density lipoprotein; NDGA, nordihydroguaiaretic acid; PBS, Dulbecco's phosphate-buffered saline pH 7.4.

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(final concentrations of 3 mM, 90 kallikrein-inhibitory units and 20 $\mu\text{g}/\text{ml}$ respectively) by sequential density-gradient ultracentrifugation in KBr solutions at 10 °C. The LDL was washed by ultracentrifugation at d 1.064 and then dialysed for 24 h against several changes of 50–100 vol. of phosphate-buffer saline (PBS) containing 1.0 mg of EDTA/ml and 0.1 mg of chloramphenicol/ml. LDL was labelled with Na^{125}I by the iodine monochloride method [13], and the iodinated LDL (specific radioactivity 50–300 d.p.m./ng of LDL protein) was separated from unreacted iodide using a column of Sephadex G-25 (PD-10; Pharmacia) followed by dialysis for 24 h against PBS containing EDTA (1.0 mg/ml) and chloramphenicol (0.1 mg/ml).

All solutions used in the isolation were deoxygenated by bubbling with argon, and all dialyses were performed under near-anoxic conditions at 4 °C in filled stoppered bottles. These procedures were adopted to minimize oxidation of the LDL during the isolation process.

Preparation of lipoxygenase inhibitors and antioxidants

Stock (1000 $\times$) solutions were prepared immediately before use in the following solvents: BW A4C, MK 886 and Revlon 5901 in dimethyl sulphoxide (DMSO); BW755C in distilled water; NDGA, BHT and Ionox 220 in ethanol. ETYA in ethanol was used at a final solvent concentration of 1% (v/v) for all systems, except for phycoerythrin fluorescence, where it was in DMSO at 0.01% (v/v).

Isolation of macrophages

Resident macrophages were obtained by peritoneal lavage of QS mice (25–35 g) with ice-cold DMEM to which was added 10 units of heparin/ml. The cells were plated in 22 mm-diam. wells (Costar) in DMEM containing 10% (v/v) heat-inactivated foetal calf serum and gentamicin (50 $\mu\text{g}/\text{ml}$) at 2×10^6 cells/ml for the cultures which were to modify LDL, and at 1×10^6 cells/well for the cultures in which uptake and degradation of LDL were to be determined. The cells were incubated at 37 °C for 2 h and then washed four times with PBS to remove contaminating non-adherent cells. Cultures were used immediately for modification of LDL, or after overnight incubation in DMEM containing 10% (v/v) foetal calf serum and gentamicin (50 $\mu\text{g}/\text{ml}$) for measurement of LDL uptake.

LDL modification

^{125}I -labelled LDL was dialysed to remove EDTA from the solution over 12 h against 2×500 vol. of PBS containing 1 g of Chelex-100 litre and then for 1 h against 500 vol. of PBS. The labelled LDL (100 $\mu\text{g}/\text{ml}$; 0.6 ml/well) was incubated for 24 h in Ham's F-10 medium, either with modifying macrophage cultures (macrophage-modified LDL) or in cell-free dishes (cell-free control LDL). The lipoxygenase inhibitors and antioxidants were added to the LDL-containing medium as stock solutions in DMSO or ethanol. Incubations were also prepared in which the same concentrations of solvent (0.1%, v/v, except for ETYA which was 1.0%) were added in the absence of inhibitors. As previously reported [11], we found that ETYA was toxic when incubated with macrophages in serum-free Ham's F-10. Lipoprotein-deficient serum was therefore included in the medium at concentrations from 0.05–0.2% (v/v). Both 0.2 and 0.1% lipoprotein-deficient serum blocked the oxidative modification, even in ETYA-free control cultures (results not shown), so that it was not possible to observe any inhibitory effects due to ETYA. Serum at 0.05% permitted modification to proceed, and protected the cultures against gross lysis (assessed by microscopic examination) at concentrations up to 1.0 μM -ETYA, though at 10 μM the cells appeared dead by the end of the modification period.

At the end of the modification period, the medium was removed and centrifuged (1000 g , 10 min) to remove any detached cells. Samples of the LDL-containing medium were diluted into fresh medium for measurement of uptake by cultured macrophages (see below).

LDL uptake and degradation by cultured macrophages

The various ^{125}I -LDLs were diluted to 10 μg of protein/ml in DMEM containing 10% (v/v) foetal calf serum and gentamicin (50 $\mu\text{g}/\text{ml}$), and incubated for 20 h (0.6 ml/well) with macrophages or with cell-free wells. This medium does not permit further macrophage-mediated modification of LDL [2]. At the end of the incubation, 0.5 ml of the medium was removed, mixed with 0.1 ml of BSA (30 mg/ml) and 1.0 ml of ice-cold 3 M-trichloroacetic acid, and centrifuged at 1500 g for 10 min at 4 °C. AgNO_3 (0.25 ml; 0.7 M) was then added to the same tube and the sample was recentrifuged. A 1.0 ml sample of the iodide-free trichloroacetic-acid-soluble supernatant was removed and its radioactivity determined.

The macrophages were washed four times with PBS and dissolved in 0.6 ml of NaOH (0.2 M) for 20 min with shaking at room temperature. Samples of 0.5 ml were removed for determination of radioactivity and protein content.

Stimulation and measurement of 5-lipoxygenase activity in cultured macrophages

Macrophage cultures (2×10^6 cells/well) were incubated with 0.5 ml of Ham's F-10 medium containing inhibitors (added as concentrated stock solutions) or solvent controls where appropriate for 10 min at 37 °C. Medium (0.5 ml) also containing inhibitor plus calcium ionophore A23187 [final concentration 2.5 μM with 0.1% (v/v) DMSO as solvent] was then added to each well and the incubation was continued for 30 min. Preliminary experiments established that these were the optimum concentration and incubation time for stimulation of 5-lipoxygenase. At the end of the incubation, samples of medium were removed and centrifuged (12000 g for 2 min) in a micro-centrifuge. LTB_4 was determined in undiluted supernatant medium samples by radioimmunoassay [14].

Inhibition of AAPH-mediated loss of phycoerythrin fluorescence

Peroxy radicals, generated in aqueous solution at a constant rate from the thermal decomposition of AAPH at 37 °C in the presence of oxygen, were used to modify the fluorescent pigment phycoerythrin. The rate of decrease in fluorescence of the molecule is related to the rate of radical generation; this can be used to investigate the activity of compounds as peroxy radical scavengers (but not as chain-breaking antioxidants, since the system is free of unsaturated lipid) [15]. The rate of loss of B-phycoerythrin (Molecular Probes, Junction City, OR, U.S.A.) fluorescence was monitored in solutions containing 15 nM-phycoerythrin and 4 mM-AAPH in 0.075 M-sodium phosphate (pH 7.0) at 37 °C. Linear decreases in fluorescence emission were measured at 565 nm with excitation at 540 nm in a Hitachi F-4010 fluorescence spectrophotometer. The ability of a range of compounds to scavenge peroxy radicals was determined by including them in this system, dissolved in DMSO or water (BW755C) to a final concentration of 10 μM . Controls included solvent alone at the same (0.1%, v/v) concentration. This concentration of DMSO alone had no significant effect on the rate of AAPH-mediated loss of phycoerythrin fluorescence. The rates of loss of fluorescence in the presence (a) and the absence (b) of the various added compounds were used to calculate the proportion of the radical production which was scavenged by each, according to the relationship: $100 \times (b-a)/b\%$.

Table 1. Effects of antioxidants and lipoxygenase inhibitors on macrophage-mediated modification of LDL to a high-uptake form

The data are the means of several separate experiments (numbers shown in parentheses), each of which included triplicate cultures for each data point. The s.d. both within each experiment and between experiments did not exceed 15 % of the means. Net modification is defined as the uptake of macrophage-modified LDL ($\mu\text{g}/20\text{ h}$ per mg of cell protein) minus the uptake rate of the corresponding cell-free control LDL (see the Experimental section). Data are expressed as percentages of the modification which occurred in the absence of added inhibitors. Separate solvent controls were used to compute this value for each inhibitor tested. ETYA-treated cultures contained 0.05 % (v/v) human lipoprotein-deficient serum.

Compound (no. of experiments)	Inhibitor concn. (μM)...	Net LDL modification (% of control activity)				
		0.05	0.1	0.5	1.0	10
BW755C (4)		66.7	3.6		1.8	0.25
BHT (1)			46.2	18.0	4.7	0.9
NDGA (4)			66.2	5.5	0.8	0
Ionox-220 (1)		78.0		22.2		
BWA4C (5)			53.4	47.1	0	0
ETYA (1)			56.2		39.3	0
Revlon 5901 (2)			89.9	96.8	88.6	72.1
MK 886 (1)			101		94.6	81.0

Copper-induced peroxidation of LDL

Peroxidation of LDL solutions ($125\text{ }\mu\text{g}/\text{ml}$) was monitored spectrophotometrically by measurement of diene conjugation at 234 nm. Oxidation was induced by the addition of $10\text{ }\mu\text{M}$ - Cu^{2+} to the same cuvette, and $4\text{ }\mu\text{M}$ -BHT was included in the reference cuvette. Antioxidants were added as ethanolic solutions [not exceeding 1 % (v/v) final concentration].

Measurement of lipid peroxyl radical scavenging

Peroxidation of a suspension of linoleic acid (52 mM) in 50 mM -phosphate (pH 7.4) was initiated with 11 mM -AAPH at $37\text{ }^\circ\text{C}$ in the cuvette of an oxygen electrode [16]. Oxygen uptake was monitored for 5 min, then the antioxidant was added. By comparing the rate of oxygen uptake in the presence and absence of antioxidant, at constant linoleic acid and AAPH concentrations, it is possible to calculate a constant, k_{AH} , which is a measure of the radical-scavenging ability of the compound [16].

RESULTS

Macrophage-mediated modification of human LDL

^{125}I -labelled human LDL was incubated for 24 h with resident peritoneal macrophage cultures under conditions which permit oxidative modification, as described in the Experimental sections. We have previously shown that normal human LDL incubated under these conditions is, after a lag period of 4–6 h, progressively modified to a 'high-uptake' form which is rapidly endocytosed by macrophages [4]. This modification is accompanied by oxidation of the LDL lipids, and the initial lag period corresponds to the time necessary for consumption of endogenous antioxidants and the onset of lipid peroxidation. In this study we assessed the modification of LDL in the presence of added compounds after a 24 h incubation with modifying macrophages. Lipoxygenase inhibitors were added as stock solutions at the beginning of the modification, and solvent controls were included. None of the solvents (DMSO, ethanol) had any detectable effect on the progress of macrophage-mediated modification, or on cell viability (determined by microscopic examination of the cultures and by recovery of cell protein from the cultures). Corresponding cell-free incubations were performed in parallel. The extent of macrophage-mediated modification was determined by measuring the rate of uptake (cell-associated plus medium content of degraded ^{125}I -LDL) of the incubated LDLs by a second set of

macrophages in medium containing 10 % serum (which prevents further oxidation of LDL). Uptake of LDL incubated in the cell-free conditions was unchanged compared with lipoprotein stored at $4\text{ }^\circ\text{C}$.

The results obtained with the complete range of agents tested are shown in Table 1. In the absence of any addition, the extent of LDL modification to a high-uptake form varied between experiments, but was usually within the range 6–26 μg of LDL protein/20 h per mg of cell protein. This variability probably represents differences in antioxidant and unsaturated lipid content between individual LDL preparations. We [4] and others [17] have previously noted that LDL preparations from individual donors vary in their susceptibility to cell-free oxidative stress. Uptake of LDL incubated in the absence of modifying macrophages (cell-free control) was less than $1\text{ }\mu\text{g}$ of LDL/20 h per mg of cell protein. The data are computed as net modification (cell-modified less cell-free control LDL uptake rates), and are listed in rank order of inhibitory activity. The rate of uptake of LDL previously modified by macrophages in the absence of additions was not affected by the inclusion of any of the compounds shown in Table 1 at $1\text{ }\mu\text{M}$ during the uptake period (results not shown). This is the maximum concentration which is likely to be carried over with modified LDL into the uptake assay. Thus the inhibitory results shown in Table 1 are unlikely to be due to direct effects of the compounds on the endocytosis of modified LDL.

All of the agents tested showed some inhibitory activity in the concentration range 0.1 – $10\text{ }\mu\text{M}$. Of these, by far the most potent was the phenyl pyrazoline BW755C, a powerful antioxidant which inhibits both cyclo-oxygenase and 5-, 12- and 15- lipoxygenases [18,19]. Its activity is unlikely to be due to inhibition of cyclo-oxygenase, since indomethacin has previously been shown to have no effect [20]. Complete inhibition of the modification process was found at $0.1\text{ }\mu\text{M}$, with near 50 % inhibition at $0.05\text{ }\mu\text{M}$ (IC_{50} value). The phenolic antioxidant BHT was also an effective inhibitor of modification, with 50 % inhibition at approx. $0.1\text{ }\mu\text{M}$. Ionox 220 and NDGA (which has both antioxidant and lipoxygenase-inhibitory properties) had similar activity to BHT. BWA4C, an acetohydroxamic acid which is a potent and relatively selective inhibitor of 5-lipoxygenase [16], gave 50 % inhibition in the range 0.1 – $0.5\text{ }\mu\text{M}$. In contrast, the 5-lipoxygenase inhibitors MK 886 and Revlon 5901 had no detectable effects at concentrations below $10\text{ }\mu\text{M}$. Probucol has previously been shown to inhibit modification, with an IC_{50} of

Table 2. Inhibition of the macrophage 5-lipoxygenase pathway

Macrophage cultures were preincubated for 10 min at 37 °C in serum-free Ham's-F10 medium containing the compounds indicated, before the addition of calcium ionophore A23187 (final concentration 2.5 μM). The incubation was continued for 30 min and then the medium was removed and assayed for LTB_4 . Controls containing appropriate solvents were included for each compound. The data are expressed as percentages of control (uninhibited) activity, and are the averages of several separate experiments, each of which included triplicate cultures for each data point. Within each experiment the S.D. of the mean did not exceed 10%; the S.D. between the means of separate experiments did not exceed 30%. Control values for A23187-stimulated LTB_4 release in the absence of inhibitors were in the range 60–350 pg/10⁶ cells

Compound (no. of experiments)	Inhibitor concn. (μM)...	LTB ₄ release (% of control activity)		
		0.1	1	10
MK 886 (1)		26.3	16.1	16.8
BWA4C (3)		34.7	0	0
Revlon 5901 (3)		50.1	35.9	71.1
NDGA (4)		66.8	15.0	4.6
ETYA (1)		79.7	69.5	61.9
ETYA/0.05 % serum (1)		106.5	87.7	77.4
BHT (2)		97.8	102	88.8
Ionox-220 (2)		121	120	108
BW755C (4)		118	124	93
Probuco1 (2)		113	109	108

less than 1 μM [20]. ETYA also inhibited macrophage-mediated modification, though at 10 μM it also showed marked evidence of cytotoxicity, even in the presence of 0.05 % lipoprotein-deficient serum.

Macrophage lipoxygenase activity and its inhibition

The activities of the compounds as inhibitors of 5-lipoxygenase were determined by measuring their effects on the release of LTB_4 from macrophages stimulated with Ca^{2+} ionophore A23187. The results are shown in Table 2.

BWA4C and MK 886 were found to be a potent inhibitors of LTB_4 synthesis in macrophages, with IC_{50} values at or below

0.1 μM . Revlon 5901 inhibited release of LTB_4 with an apparent IC_{50} of 0.1 μM , but full inhibition was not achieved at 1 μM . At very high concentrations (10 μM) an increase in LTB_4 release into the medium was found. NDGA showed moderate potency, with an IC_{50} of between 0.1 and 0.5 μM , while BW755C showed little inhibitory activity at concentrations up to 10 μM . These results are closely similar to data previously reported for human leucocytes and whole blood [16,21,22]. BW775C has inhibitory effects only at higher concentrations, with an IC_{50} of 30 μM in leucocytes [18]. The antioxidants BHT, Ionox-220 and Probuco1 had no effect on 5-lipoxygenase activity. ETYA was less potent than anticipated, with an IC_{50} in excess of 10 μM , probably because of its toxicity in this cell type.

Radical-scavenging and chain-breaking antioxidant activities

The ability of molecules to inhibit the loss of fluorescence of phycoerythrin in the presence of AAPH has been developed as an assay of their ability to scavenge aqueous peroxy radicals. Since the mechanism of initiation of cell-mediated oxidative modification of LDL may involve a water-soluble peroxy radical, this system is one appropriate method for studying the antioxidant activity of inhibitors of LDL modification. The activity of the compounds tested is shown in Table 3. All of the compounds tested protected phycoerythrin against AAPH-dependent loss of fluorescence, though there was a wide spectrum of activity. The most potent was BW755C, and BWA4C, BHT and ETYA were also good radical scavengers. NDGA and Probuco1 were less effective, and Revlon 5901, MK 886 and Ionox-220 had only minor activity.

It has been shown that low concentrations of Cu^{2+} stimulate peroxidation of LDL. The peroxidation reaction apparently causes modification of the apo B molecule, such that it becomes a ligand for macrophage 'scavenger' receptors. It is therefore a useful *in vitro* model for the processes which may lead to oxidative modification of LDL *in vivo*. The progress of the reaction can be monitored in the early stages by measuring the increase in absorbance at 234 nm, which has been ascribed to the formation of conjugated dienes in the sample. This system has been characterized by Esterbauer *et al.* [17], and we have modified it to provide an assay for comparison of compounds as antioxidants. The time course of a typical reaction is shown in Fig. 1. After a lag period of approx. 30 min, an increase in absorbance at 234 nm occurs, which reaches a maximum after approx.

Table 3. Measurement of antioxidant activity

For the phycoerythrin assay, agents were added to the assay system as solutions in DMSO. Final concentrations of agent and DMSO were 10 μM and 0.1 % (v/v). Inhibition is measured relative to a control rate in the presence of DMSO. Data are means $\pm$ S.D. of triplicate determinations. The IC_{50} is the concentration of agent (μM) required to increase the time for half-maximal absorbance at 234 nm by 60 min. Data are means $\pm$ S.D. of three separate determinations. The constant k_{AH} is a measure of the radical-scavenging ability of the compound in AAPH-stimulated peroxidation of linoleic acid [16]. The data are the means $\pm$ S.D. of triplicate determinations.

Compound	Phycoerythrin assay (% inhibition)	Cu^{2+} -mediated oxidation (IC_{50})	Linoleate oxidation (k_{AH})
BW755C	86.76 $\pm$ 0.10	1.44 $\pm$ 0.32	4.03 $\pm$ 0.05
BWA4C	61.50 $\pm$ 0.03	7.54 $\pm$ 0.30	0.14 $\pm$ 0.02
ETYA	59.44 $\pm$ 3.18	None*	None*
BHT	48.39 $\pm$ 2.06	1.03 $\pm$ 0.29	0.75 $\pm$ 0.10
NDGA	18.49 $\pm$ 0.50	0.98 $\pm$ 0.33	0.27 $\pm$ 0.04
Probuco1	15.83 $\pm$ 1.21	1.69 $\pm$ 0.16	0.27 $\pm$ 0.06
Revlon 5901	7.07 $\pm$ 0.41	None*	None*
MK 886	3.46 $\pm$ 6.00	None*	None*
Ionox-220	0.30 $\pm$ 4.65	1.15 $\pm$ 0.15	1.46 $\pm$ 0.28

* None: No antioxidant activity was detected at concentrations up to 10 μM .

Native LDL was added to the reference cuvette of a spectrophotometer at a final concentration of 125 $\mu\text{g/ml}$ in the presence of 4 μM -BHT. Peroxidation of LDL was initiated by the addition of 10 μM - Cu^{2+} .

Fig. 2. Conjugated diene formation in the Cu^{2+} -mediated peroxidation of LDL

DISCUSSION

Although cell-mediated oxidative modification of LDL has been the subject of intense scrutiny in recent years, the mechanism by which cells such as macrophages initiate LDL oxidation still remains unclear. We were interested in recent reports suggesting that lipoxygenase activity may be important in this process, since macrophages are capable of the synthesis of 5-, 8-, 9-, 12- and 15-hydroxyeicosatetraenoic acids when incubated with exogenous arachidonic acid [23]. The difficulty in establishing that lipoxygenase activity is an essential component of macrophage-mediated modification is that many lipoxygenase inhibitors are also antioxidants. It is therefore necessary to be able to distinguish between these two properties when the activities of lipoxygenase inhibitors are studied. For this reason we undertook to study the effects of several compounds on macrophage-mediated modification of LDL and to compare them with the activities of the same compounds as antioxidants and lipoxygenase inhibitors. In this study we have concentrated mainly on inhibition of 5-lipoxygenase, for which inhibitors and a reliable assay of enzyme activity were readily available.

In measuring the antioxidant activity of the compounds, we found that it was necessary to use more than one system. We first tested the compounds in assays which are designed to identify compounds as peroxy radical scavengers. These involved the thermal decomposition of the free radical initiator AAPH, which in the presence of oxygen produces aqueous peroxy radicals at a constant rate. The first assay is based on the detection of chemical damage to the protein B-phycoerythrin, and its prevention by compounds able to scavenge aqueous peroxy radicals. The data indicated that BW755C, BWA4C, ETYA and BHT were all effective aqueous peroxy radical scavengers. NDGA and Probulol were 3–4-fold less active, and Revlon 5901, MK 886 and Ionox-220 had little or no activity.

This assay is capable of detecting compounds which react with aqueous peroxy radicals, but does not necessarily identify chain-breaking antioxidants. The latter must be able to react with lipid phase radicals. Further, if the antioxidant radical species is capable of re-initiating lipid peroxidation through hydrogen abstraction, then no chain-breaking antioxidant activity will be exhibited. For this reason we have also tested the ability of the agents to inhibit AAPH-dependent peroxidation of linoleic acid, using oxygen uptake as an index of peroxidation. In this system, the lipoxygenase inhibitors BW755C, BWA4C and NDGA were all peroxy radical scavengers, whereas Revlon 5901, MK 886 and ETYA were not. This result is consistent with the structures of the compounds (Fig. 3). The classical chain-breaking antioxidants BHT and Ionox-220 also show the expected activity. The poor activity of Ionox-220 in the phycoerythrin assay may be a consequence of its low aqueous solubility.

The precise mechanism by which Cu^{2+} induces peroxidation of LDL has yet to be established. For this reason we cannot, *a priori*, ascribe the inhibition of conjugated diene formation by the compounds tested in this assay solely to peroxy radical scavenging. For example, it is possible that some compounds such as the lipoxygenase inhibitor BWA4C chelate the copper such that it is unable to initiate peroxidation. However, we have previously shown that *N*-hydroxy compounds such as BWA4C are able to scavenge peroxy radicals in a metal-independent system [16] such as the AAPH-dependent oxidation of linoleate (Table 3). Other mechanisms distinct from radical scavenging are also possible.

All of the compounds showing activity in inhibiting conjugated diene formation in the Cu^{2+} -induced peroxidation of LDL were also active as peroxy radical scavengers. There is not, however, a good correlation in the activity of individual compounds between these assays. Variations in the relative activities of the compounds between these assays may relate to differences in their partition between aqueous and lipid phases and their reactivity as chain-breaking antioxidants by reaction with lipid radicals (which would also contribute to the depression of oxygen uptake by LDL). For example, the water-soluble phenyl pyrazoline BW755C was more potent in protecting phycoerythrin against attack than in suppressing Cu^{2+} -induced peroxidation of LDL lipids. This is probably related to the different mechanisms of the initiation of peroxidation by AAPH and copper, and the phase (aqueous versus lipid) in which the majority of the radical scavenging occurs.

It is not surprising that some 5-lipoxygenase inhibitors (NDGA, BW755C, BWA4C) are also antioxidants, since oxidants are produced during this enzymic reaction and these compounds are thought to interact with the active site of the enzyme [18]. Rather unexpectedly, ETYA was apparently also a good peroxy radical scavenger in the phycoerythrin system. This is the first report of radical-scavenging activity by ETYA of which we are aware. The mechanism by which ETYA could interact with peroxy radicals is not clear. Revlon 5901 and MK 886 are, however, important exceptions to the general trend. These lipoxygenase inhibitors, which act to inhibit translocation of 5-lipoxygenase to the cell membrane, showed no evidence of antioxidant activity in any of the systems used. Since these two inhibitors are mechanistically distinct from BWA4C, it is perhaps not surprising that the dose-dependence for the inhibition of LTB_4 release is different from that found for compounds such as BWA4C and NDGA, which interact directly with the enzyme itself.

The lipoxygenase inhibitors (BWA4C, MK 886, Revlon 5901, NDGA and ETYA) are able to inhibit macrophage 5-lipoxygenase, as shown in Table 2. The antioxidants BHT, Ionox-220 and Probucol did not inhibit 5-lipoxygenase at the concentrations

tested. BW755C, though a lipoxygenase inhibitor, had little activity in this system up to a concentration of $10\text{ }\mu\text{M}$, which is consistent with data for neutrophils where an IC_{50} of $30\text{ }\mu\text{M}$ was measured [18]. However, several of these compounds (particularly BW755C) were extremely potent as inhibitors of macrophage-mediated LDL modification. Thus the group of compounds studied contains antioxidants which do not inhibit 5-lipoxygenase (BHT, Ionox-220, Probucol), lipoxygenase inhibitors which are also antioxidants (BW755C, NDGA and BWA4C) and compounds which are 5-lipoxygenase inhibitors but not antioxidants (Revlon 5901 and MK 886).

We propose that the effects of these compounds on the macrophage-dependent modification of LDL allows a discrimination between the roles of antioxidants and lipoxygenase enzymes in this process. This is despite the fact that total inhibition of 5-lipoxygenase is not achieved with either Revlon 5901 or MK 886. It is extremely unlikely that the 64% inhibition of 5-lipoxygenase achieved with Revlon 5901 or the 84% inhibition found with MK 886 would have no effect on macrophage modification of LDL if 5-lipoxygenase were involved. It has been shown *in vitro* that chemical peroxidation, lipoxygenase treatment and macrophage-mediated modification of LDL all result in transformation of the particle to forms recognized by macrophage scavenger receptor(s). Our results suggest that only compounds acting as antioxidants can inhibit the macrophage-mediated modification of LDL, and that lipoxygenase inhibition alone, as found with Revlon 5901 and MK 886, is not sufficient. We suggest that complete lack of the ability of these latter two compounds to prevent macrophage-mediated modification of LDL at concentrations where extensive inhibition of 5-lipoxygenase is achieved is strong evidence that this enzyme is not essential for the modification process. Since both of these compounds are inhibitors of 5-lipoxygenase activator protein [24], which is specific for 5-lipoxygenase, it cannot be argued that peroxy radicals are likely to leak from the inhibited enzyme. Furthermore, the addition of MK 886 to macrophage cultures has no effect on the rate of release of 15-hydroxyeicosatetraenoic acid (W. Jessup, unpublished work), indicating that blockage of 5-lipoxygenase is probably not coupled to an increase flux of substrate though alternative lipoxygenase pathways.

Our conclusion is further supported by examination of the concentration-dependence of BWA4C activity as a lipoxygenase inhibitor and in suppression of macrophage-mediated modification of LDL. At concentrations ($> 0.1\text{ }\mu\text{M}$) where 5-lipoxygenase was completely inhibited, substantial cell-mediated LDL modification still occurred.

We suggest that, while lipoxygenase activity may have a pro-oxidant effect in enhancing LDL oxidation, and this possibility cannot be discounted as a contributory factor in cell-mediated oxidation under some circumstances, it is not a necessary condition for the system of macrophage-mediated modification *in vitro* that we have used in this study. The possibility remains that, although 5-lipoxygenase does not play a role in LDL modification, other enzymes, such as 12- and 15-lipoxygenases, are of greater importance. These are certainly present in resident macrophages and under unstimulated conditions they may represent a substantial proportion of total lipoxygenase activity [23]. To elicit their role in macrophage-mediated modification it will be necessary to obtain selective inhibitors without antioxidant properties, as we have done here for 5-lipoxygenase. MK 886 has no activity against macrophage 15-lipoxygenase (W. Jessup, unpublished work). Thus we are presently unable to comment on the possibility that this enzyme may be essential.

The use of ETYA as an inhibitor in studying the role of other lipoxygenases must be treated with caution, since this compound shows some evidence of radical-scavenging properties and in

macrophages is extremely toxic. This latter property has recently been separately reported by another group [25]. Interestingly, ETYA shows no activity characteristic of a chain-breaking antioxidant, and is unlikely therefore to prevent the modification of LDL by inhibiting propagation within the lipid phase of the particle. Its reactivity with peroxyl radicals, as implied by the phycoerythrin assay, raises the possibility that ETYA may intercept and scavenge the initiating oxidant released by the macrophage and so prevent the initial step in the peroxidation of LDL. It does however remain possible that its effects on LDL oxidation by macrophages are directly related to inhibition of 12- or 15-lipoxygenase. ETYA is known to be a better inhibitor of 15- than 5-lipoxygenase [22], and direct determination of the IC_{50} for ETYA inhibition of macrophage 12- and 15-lipoxygenases would be useful. Even so, its toxicity and lack of specificity limit the conclusions which can be made based on this compound alone.

In summary, it is suggested that although 5-lipoxygenase activity may have a synergistic effect in promoting macrophage-mediated LDL oxidation, it is not a necessary component. This then implies that oxidants generated and released by the macrophage may initiate peroxidation in the LDL particle. Characterization of these species is clearly important in clarifying this important biological model of oxidative modification of LDL.

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