Human macrophage-mediated oxidation of low-density lipoprotein is delayed and independent of superoxide production

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There is growing evidence that oxidatively modified low-density lipoprotein (LDL) accumulates in the atherosclerotic intima of arteries. Cells present in the intima (including the monocyte/macrophage) are capable of oxidizing LDL *in vitro*, but the mechanisms by which this occurs are unknown. Several reports have claimed a crucial role for superoxide as a cell-derived radical species capable of enhancing the rate of LDL oxidation. We have used a sensitive h.p.l.c. system with chemiluminescence detection to measure LDL cholesteryl ester hydroperoxides at early stages of LDL oxidation. During the initial stages of LDL oxidation, there is at least a 2 h delay before human monocyte-derived macrophages enhance this process. Stimulation of these

cells to produce large fluxes of superoxide does not increase the rate of LDL oxidation or decrease the delay of its onset. Prior exposure of LDL to a high flux of superoxide does not increase its susceptibility to oxidation by human monocyte-derived macrophages. We also show that the thiobarbituric acid-reactive substances (TBARS) assay does not always correlate with more direct methods of assessing LDL oxidation and confirm recent reports that superoxide dismutase only partially inhibits cell-mediated LDL oxidation. We conclude that superoxide does not play a major role in human monocyte-derived macrophage-mediated LDL oxidation under the conditions that we describe.

INTRODUCTION

The monocyte/macrophage is quantitatively one of the most important cell types in the intima of arteries during the development of atherosclerotic lesions [1] and is present at all stages of atherogenesis [2]. There is strong evidence to show that the peripheral-blood monocyte is capable of crossing the intact endothelium [3], and it has been proposed that the development of atherosclerosis commences with this infiltration of monocytes to the sub-endothelial space and the subsequent endocytosis of intimal low-density lipoprotein (LDL) [4]. Oxidative modification of intimal lipoproteins is suspected to be an essential component of this atherogenic process [5].

Although a number of studies have found oxidized LDL in atherosclerotic plaque [6,7], the mechanism by which this is produced is at present unknown. All of the cell types present in the developing lesion, including macrophages [8], endothelial cells [9], smooth-muscle cells [10] and lymphocytes [11], have the ability to accelerate the oxidation of LDL *in vitro*. In all cases, such cell-mediated oxidation appears to be transition-metal-dependent, but there is no consensus on the mechanisms by which cells promote oxidation. Production of arachidonic acid oxygenation products in the plasma membrane by lipoxygenase has been suggested as a means by which cells could promote LDL oxidation [12–15]. This process would rely on transfer of these plasma-membrane lipids to the LDL particle or release of lipoxygenase to the extracellular environment, where it could utilize LDL lipid as substrate. However, a number of groups have presented evidence which disagrees with these mechanisms [16–18].

Several years ago Heinecke et al. [10] proposed that production of cellular reductants, such as thiols, could reduce oxygen to

form superoxide radicals ($O_2^{\cdot-}$), which could enhance LDL oxidation in the presence of transition metals, presumably via a redox-cycling mechanism. This could accelerate oxidation by facilitating breakdown of LDL lipid hydroperoxides [19]. More recently, it has been proposed that transition metals may react directly with thiols to form thiyl radicals, which may oxidize LDL lipids [20]. This mechanism would also maintain transition metals in a redox-active state, and thus enhance LDL oxidation by increasing the rate of breakdown of lipid hydroperoxides. Another possible mechanism of cell-mediated LDL oxidation involves cellular production of highly reactive radicals such as the hydroxyl radical ($HO^{\cdot}$). Superoxide can dismutate to form H_2O_2 , which, in the presence of transition metals, could generate $HO^{\cdot}$. Although $O_2^{\cdot-}$ acts as an electron donor (reductant) in most redox reactions likely to occur under physiological conditions, $HO^{\cdot}$ is a powerful oxidizing agent. Although $O_2^{\cdot-}$ could theoretically produce $HO^{\cdot}$ extracellularly, there is little evidence that this radical is responsible for cell-mediated LDL oxidation. In addition, mannitol (a $HO^{\cdot}$ scavenger) does not block endothelial-cell-mediated LDL oxidation [21]. $O_2^{\cdot-}$ produced by γ -radiolysis has been shown to increase marginally the rate of LDL oxidation; however, it does so without generating a form recognized by macrophage scavenger receptors [22]. The implication of a major role for $O_2^{\cdot-}$ in cell-mediated oxidation has mainly arisen from the observation that cells stimulated to produce $O_2^{\cdot-}$ produce higher concentrations of lipid-peroxidation products, judged by thiobarbituric acid-reactive substances (TBARS) in culture supernatants containing LDL, and the observation that superoxide dismutase (SOD) has the ability to inhibit TBARS generation to varying degrees [23–25]. However, the TBARS assay is an indirect measure of lipid peroxidation [26], which detects only a very small proportion of LDL-oxidation

Abbreviations used: CE, cholesteryl ester; CE-OOH, CE hydroperoxide; HBSS, Hanks' balanced salt solution; $HO^{\cdot}$, hydroxyl radical; LDL, low-density lipoprotein; MDA, malondialdehyde; MDM, monocyte-derived macrophages; $O_2^{\cdot-}$, superoxide radical; PBMC, peripheral-blood mononuclear cells; PMA, phorbol 12-myristate 13-acetate; SOD, superoxide dismutase (EC 1.15.1.1); TBA, thiobarbituric acid; TBARS, TBA-reactive substances.

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products [27], and the use of SOD in metal-mediated LDL-oxidation studies may lead to non-specific antioxidant activity, due to the metal chelating properties of this enzyme [28,29]. Thus the present evidence of $O_2^{\cdot-}$ involvement in cell-mediated oxidation is inconclusive.

In the present study we have used highly purified human monocyte-derived macrophages (MDM) to oxidize LDL in Ham's F10 medium (composition includes $3 \mu\text{M}$ Fe and $0.01 \mu\text{M}$ Cu). The respiratory-burst oxidase system in these cells was triggered to produce high fluxes of $O_2^{\cdot-}$. LDL oxidation was assessed by using a sensitive h.p.l.c. method to measure depletion of LDL cholesteryl esters (CEs) and the simultaneous formation of CE hydroperoxide (CE-OOH) with post-column chemiluminescence, both during and after stimulation of the MDM respiratory burst. We show that there is a lag time of at least 2 h before MDM enhance LDL oxidation compared with media alone, and we find no evidence that increased $O_2^{\cdot-}$ production during or after this period increases the rate of LDL oxidation in Ham's F10 medium. In addition, these results are compared with TBARS detected under the same conditions (and up to 24 h incubation) and show that, at least under the conditions described, the TBARS assay may give misleading results, and is therefore not the method of choice to determine LDL oxidation.

The conclusions drawn from the present study contrast with the relatively few previous studies which have relied on less-sensitive methods to assess the role of $O_2^{\cdot-}$ in monocyte- (e.g. [23]) or peripheral-blood-mononuclear-cell (PBMC)-mediated LDL oxidation (e.g. [25]). In addition, our data relate to the function of human MDM, which have been less well studied previously.

MATERIALS AND METHODS

Materials

RPMI 1640, Hanks' balanced salt solution (HBSS), PBS, copper/zinc-containing SOD (catalogue no. S-2515), phorbol 12-myristate 13-acetate (PMA), cytochrome *c* (catalogue no. C-2037) and EDTA were all obtained from Sigma. Ham's F10 medium (catalogue no. 320-1550AJ) was from Gibco. PMA was dissolved in dimethyl sulphoxide and stored at a stock concentration of 10 mg/ml at -20°C . SOD was dissolved in sterile nanopure water and stored at -20°C at a stock concentration of 1 mg/ml (after filter sterilization; $0.22 \mu\text{m}$ pore size) for up to 5 months. H.p.l.c.-grade hexane and methanol were from Mallinckrodt. *t*-Butyl alcohol and ethanol (both analytical grade) were from Rhône-Poulenc and BDH respectively. White-cell concentrates (< 24 h *ex vivo*) and human serum (for tissue culture) were kindly provided by the N.S.W. Red Cross Blood Transfusion Service, Sydney, Australia.

LDL isolation

Human LDL (density ≈ 1.06 g/ml) was isolated from non-fasted normolipidaemic volunteers by using a rapid procedure to minimize oxidative damage to the LDL [30]. Briefly, plasma was separated from EDTA-anticoagulated blood (EDTA- K_3 vacutainers; Becton Dickinson) by centrifugation at 1000 *g* for 15 min at 15°C . The plasma was then adjusted to a density of 1.24 g/ml by addition of solid KBr. The LDL was subsequently isolated in a two-step gradient by ultracentrifugation for 2 h at 15°C in a Beckman TL-100.4 rotor and TL-100 benchtop centrifuge [30]. The isolated LDL was then dialysed for 18 h against four changes each of 100 vol. of deoxygenated PBS

containing 100 mg/l chloramphenicol (Boehringer, Mannheim, Germany) and 5 g/l Chelex-100 (Bio-Rad). Chelex was omitted in the last dialysis step. The LDL was then filter-sterilized ($0.45 \mu\text{m}$ pore size) and used immediately in experiments. LDL prepared by this method consistently contained only low levels of CE-OOH (45 ± 38 pmol/mg of LDL protein; mean $\pm$ S.D., $n = 10$), approx. 1 CE-OOH molecule for every 43 LDL particles present on average. LDL protein concentration was assayed by the bicinchoninic acid method (Sigma, catalogue no. TPRO562) with BSA as a standard.

Isolation and culture of MDM

Monocytes were isolated from white-cell concentrates by using countercurrent centrifugal elutriation essentially as described by Stevenson [31]. The elutriation system consisted of a Beckman JE-6 rotor in a Beckman J2-21M/E centrifuge equipped with strobe assembly. Flow through the system was controlled with a Cole Parmer 7545 Masterflex pump and 7021-20 head. The isolation procedure was performed at 21°C . White-cell concentrates were diluted 1:2 in elutriation medium (HBSS minus Ca^{2+} and Mg^{2+} , but containing 100 mg/l EDTA and 1% non-fasted human serum). PBMC were isolated from white-cell concentrates after underlaying 30 ml of diluted white cell concentrate with 15 ml of Lymphoprep (Nycomed, Oslo, Norway) and centrifuging at 400 *g* for 40 min at 21°C . The harvested PBMC were then washed by spinning in elutriation medium to remove any residual Lymphoprep and were loaded into the elutriation chamber in a final volume of 50 ml ($\sim 1 \times 10^9$ PBMC). PBMC were loaded at a rotor speed of 2020 rev./min and an initial flow rate of 9.0 ml/min, which was increased by 0.5 ml/min every 10 min. With this protocol, platelets were eluted immediately, followed by lymphocytes, at a flow rate from ~ 11.0 to ~ 12.5 ml/min, and a mixed lymphocyte/monocyte fraction (with increasing monocyte purity) up to ~ 15.0 ml/min. After the 15 ml/min fraction had been eluted, the flow rate was increased to 40 ml/min to elute the remaining purified monocyte fraction from the chamber. The purified monocyte fraction was $> 95\%$ esterase-positive (Sigma, catalogue no. 181B) and $> 99\%$ viable as judged by Trypan Blue exclusion.

The monocytes were then washed in RPMI 1640 with no additives and adhered in 22 mm-diameter tissue-culture wells (Costar) at a concentration of 1×10^6 cells/well in 1 ml of RPMI 1640 for 90 min at 37°C . This medium was replaced with 1.5 ml of RPMI 1640 with 10% (v/v) heat-inactivated human serum, 20 mM glutamine, 100 i.u./ml penicillin and 100 $\mu\text{g}/\text{ml}$ streptomycin. The adhered monocytes were left to differentiate for 6 days at 37°C in 5% CO_2 in air, with changes of medium on days 3 and 5. The day-6 MDM were $> 99\%$ esterase-positive and routinely yielded 150–200 μg of protein/well. All media used for monocyte elutriation and culture were Zetapore-filtered (CUNO, Meriden, CT, U.S.A.) and were shown to be endotoxin-free (< 20 pg of lipopolysaccharide/ml) by using the *Limulus* amoebocyte assay (Associates of Cape Cod, American Diagnostica, Greenwich, CT, U.S.A.).

LDL modification

MDM were washed 3 times with PBS at 37°C before addition of LDL at a concentration of 100 $\mu\text{g}/\text{ml}$ in Ham's F10 or RPMI 1640. SOD and PMA were added to give final concentrations of 10 and 4 $\mu\text{g}/\text{ml}$ respectively where appropriate, in a final volume of 0.6 ml/well. Solvent controls were also included. LDL modification was performed at 37°C in 5% CO_2 in air. Supernatants (600 μl) were removed at the desired time point and

added to tubes containing concentrated EDTA and butylated hydroxytoluene (6 μl of each) to give a final concentration of 20 μM for each of these compounds. A 200 μl portion of this supernatant was used for h.p.l.c. determination of LDL neutral lipids and CE-OOH, and 300 μl samples were assayed for TBARS.

TBARS assay

Measurement of TBARS in culture supernatants was performed by the method of Schuh et al. [32] with minor modifications. Supernatants were centrifuged for 2 min at 14000 rev./min in an Eppendorf microfuge to remove any cells. A 300 μl portion was then mixed with 750 μl of 20% trichloroacetic acid, followed by 750 μl of 1% thiobarbituric acid (TBA). After incubation for 45 min at 100 °C, the mixture was cooled on ice, and then centrifuged for 15 min at 3000 rev./min at 10 °C. Fluorescence was measured by using 532 nm excitation/553 nm emission. Malondialdehyde (MDA) standards were prepared by using 1,1,3,3-tetramethoxypropane in Ham's F10 medium. As substances other than MDA are known to react with TBA [26], results are expressed as MDA equivalents/mg of LDL protein.

H.p.l.c. analysis of CEs and CE-OOH

A 200 μl portion of the LDL-containing culture supernatant was extracted into 2 ml of methanol plus 10 ml of hexane, as described by Sattler et al. [30] except that the methanol was not acidified. Subsequently, 9 ml of the hexane extract was evaporated under vacuum and redissolved in 180 μl of ethanol. Unoxidized neutral lipids and CE-OOH were separated on an LC-18 column (Supelco; 25 cm $\times$ 0.46 cm, with 5 cm guard column) with *t*-butyl alcohol/methanol (1:1, v/v) as mobile phase and detected by u.v. absorbance at 210 nm and post-column chemiluminescence respectively, as originally described by Yamamoto et al. [33], with the modifications described by Sattler et al. [30]. CE-OOHs were quantified by using cholesteryl linoleate (CE18:2) hydroperoxide standards, which were prepared by addition of 2,2'-azobis(2,4-dimethylvaleronitrile) (Polysciences Inc., Warrington, PA, U.S.A.) to CE18:2 (Sigma) as described by Yamamoto et al. [33]. CEs were expressed as a percentage of those originally present in the unoxidized LDL.

Superoxide production by MDM

Superoxide production was measured as the SOD-inhibitable reduction of ferricytochrome *c* [34]. MDM, cultured as described above, were washed three times with PBS at 37 °C, then incubated in 1 ml of HBSS containing 80 μM cytochrome *c* in the absence or presence of PMA (4 $\mu\text{g}/\text{ml}$). Parallel incubations were performed in the presence of SOD (10 $\mu\text{g}/\text{ml}$). The culture supernatants were placed on ice after incubation (usually 90 min) and were centrifuged to remove cells. SOD-inhibitable cytochrome *c* reduction was measured by the A_{550} . Results are expressed as nmol of $\text{O}_2^{\cdot -}$ /90 min per 10^6 cells, or nmol of $\text{O}_2^{\cdot -}$ /30 min per 10^6 cells for the kinetic studies.

Statistical analysis

Statistical significance was determined by using Student's *t* test for unpaired data where appropriate. A *P* value < 0.05 was considered significant.

RESULTS

Superoxide release by MDM

In order to study the effect of $\text{O}_2^{\cdot -}$ on LDL modification, the ability of MDM to produce $\text{O}_2^{\cdot -}$ was first examined. PMA, a potent stimulant for the respiratory-burst oxidase of these cells, stimulated production of 13.5 ± 4.9 nmol of $\text{O}_2^{\cdot -}$ /90 min per 10^6 cells (mean $\pm$ S.D.) in 9 independent experiments. This represented an average 18-fold increase over the levels produced by non-stimulated cells during the first 90 min of the respiratory burst. Figure 1 shows that the $\text{O}_2^{\cdot -}$ production rate is maximal within 30 min of stimulation and declines slowly over the next 3–4 h. These results, together with results from two additional independent experiments, show that $\text{O}_2^{\cdot -}$ production returns to near baseline levels at about 4–6 h after stimulation (results not shown) and thus allow the calculation that, over a 24 h incubation, MDM which are stimulated with PMA produce at least twice the total $\text{O}_2^{\cdot -}$ flux of non-stimulated MDM. It is important to note that, although the rate of $\text{O}_2^{\cdot -}$ production is declining by 2 h after PMA addition, there is still substantial $\text{O}_2^{\cdot -}$ production after 2 h, which accounts for between ~10 and ~50% of the total PMA-generated $\text{O}_2^{\cdot -}$ flux. SOD used at 10 $\mu\text{g}/\text{ml}$ inhibited $81 \pm 7\%$ (mean $\pm$ S.D., *n* = 9) of the PMA-stimulated cytochrome *c* reduction in these experiments.

LDL CE-OOH formation during PMA-stimulated respiratory burst in Ham's F10 medium

Under the conditions that we describe, LDL cholesteryl linoleate (CE18:2) and cholesteryl arachidonate (CE20:4) are quantitatively major substrates for LDL lipid oxidation. Measurement of the loss of CEs is therefore a direct indicator of LDL oxidation. In addition, h.p.l.c. with post-column chemiluminescence detection allows detection of CE-OOH in the pmol range [33] and is particularly useful when determining oxidative damage to the LDL at very early stages; even before loss of CE is detectable by u.v. (210 nm) monitoring, CE-OOH formation can be measured. Figure 2 shows that peroxidation of LDL occurs in this system from the earliest time assayed (30 min) and

Figure 1 Time course of PMA-stimulated MDM respiratory-burst activity

MDM (1×10^6) were incubated at 37 °C with 80 μM cytochrome *c* in HBSS in the absence (○) or presence (●) of PMA at 4 $\mu\text{g}/\text{ml}$. The resulting $\text{O}_2^{\cdot -}$ production was determined by measuring SOD-inhibitable reduction of cytochrome *c* for the indicated times as described in the Materials and methods section. The inset illustrates the decrease in the rate of $\text{O}_2^{\cdot -}$ production after the initial 30 min interval. The points on the inset represent $\text{O}_2^{\cdot -}$ production over the preceding 30 min interval. All points are single determinations from one experiment, and are representative of three independent experiments.

Figure 2 LDL CE-OOH formation during early stages of LDL oxidation in Ham's F10 medium

LDL at 100 $\mu\text{g/ml}$ was incubated with MDM ($\square$), MDM stimulated to produce $\text{O}_2^{\cdot-}$ by addition of PMA at 4 $\mu\text{g/ml}$ ($\bullet$), or in Ham's F10 medium alone ($\square$). At the times indicated, the LDL was extracted from medium and assayed for CE-OOH by using h.p.l.c. with chemiluminescence detection as described in the Materials and methods section. All points are means $\pm$ S.D. of three independent experiments, each performed in duplicate or triplicate.

Figure 3 LDL CE-OOH formation during advanced stages of incubation in Ham's F10 (a) or RPMI 1640 (b) media

LDL at 100 $\mu\text{g/ml}$ was incubated with MDM ($\blacksquare$), MDM stimulated to produce $\text{O}_2^{\cdot-}$ by addition of PMA at 4 $\mu\text{g/ml}$ ($\boxtimes$), or in medium alone ($\square$), in either Ham's F10 (a) or RPMI 1640 (b) medium. After 6 h or 24 h the LDL was extracted from the medium and assayed for CE-OOH by using h.p.l.c. with chemiluminescence detection as described in the Materials and methods section. All points are means $\pm$ S.D. of triplicate determinations from one experiment and are representative of three independent experiments.

proceeds linearly for 2 h. When PMA is added to MDM to stimulate the respiratory burst (i.e. to maximize the rate of $\text{O}_2^{\cdot-}$ production), there is no increase in the amount of LDL CE-OOH detected during this period. There is also no difference between the LDL CE-OOH content in cell-free controls and the MDM-

Figure 4 LDL CE depletion during advanced stages of LDL oxidation in Ham's F10 medium

LDL at 100 $\mu\text{g/ml}$ was incubated with MDM (MDM), MDM stimulated to produce $\text{O}_2^{\cdot-}$ by addition of PMA at 4 $\mu\text{g/ml}$ (MDM + PMA), or in Ham's F10 medium alone (Cell-free). After 6 h and 24 h the LDL was extracted from medium and assayed for cholesterol arachidonate (CE20:4; $\boxtimes$) and cholesteryl linoleate (CE18:2; $\blacksquare$) by using h.p.l.c. with u.v. (210 nm) detection as described in the Materials and methods section. Results are expressed as a percentage of the CE concentration present in the original (non-oxidized) LDL. All points are means $\pm$ S.D. of triplicate determinations from one experiment and are representative of three independent experiments.

containing incubations during this period. These results are consistent with previous reports which have used less sensitive methods to assess cell-mediated LDL oxidation, but have shown an apparent lag phase up to 3–4 h before cells measurably enhance the rate of oxidation compared with cell-free controls [29]. There was no detectable loss of CE18:2 or CE20:4 at these time points (results not shown), which is likely to be due to the difference in sensitivity between u.v. (210 nm) and chemiluminescence detection systems.

The issue of different sensitivities between detection of CE-OOH and CE is further illustrated when one considers that the initial CE20:4 and CE18:2 are typically $\approx 13 \mu\text{M}$ and $\approx 120 \mu\text{M}$ respectively, i.e. 1330 nmol of CE substrate/mg of LDL protein. Thus an approximate estimate (not allowing for subsequent reduction of CE-OOH to cholesteryl ester hydroxide, and assuming that only one molecule of CE-OOH is formed per molecule of CE oxidized) would predict that only 1 in 166 of the major CE substrates have been oxidized after 2 h incubation in Ham's F10 medium ($\sim 8 \text{ nmol}$ of CE-OOH/mg of LDL protein). Such loss is not detectable by using the u.v. (210 nm) system. The mean CE-OOH concentration for LDL at the zero time point for the three experiments represented in Figure 2 was $36 \pm 30 \text{ pmol/mg}$ of LDL protein (mean $\pm$ S.D., $n = 3$). We concluded that LDL which is exposed to high fluxes of MDM-derived $\text{O}_2^{\cdot-}$ is no more oxidized during this period (0–2 h) than is LDL incubated with either non-stimulated MDM or in Ham's F10 medium alone. However, cell-mediated LDL oxidation could be observed at later time points.

Effect of $\text{O}_2^{\cdot-}$ production on LDL oxidation after respiratory-burst activity has subsided

To assess the effect of $\text{O}_2^{\cdot-}$ production on LDL oxidation after respiratory-burst activity has subsided, we measured CE-OOH production over longer periods up to 24 h. These experiments were conducted to ensure that there was no 'sensitization' of the LDL to oxidation during the respiratory burst, for example by depleting endogenous antioxidants [22]. Figure 3(a) shows that, at 6 h after addition of PMA, LDL which has been exposed to a

stimulated flux of $O_2^{\cdot-}$ in Ham's F10 medium still does not contain significantly higher amounts of CE-OOH compared with LDL incubated with non-stimulated MDM. However, at 6 h, the LDL incubated in the presence of both stimulated and non-stimulated MDM clearly contained higher levels of CE-OOH when compared with cell-free controls. Figure 4 shows the corresponding levels of LDL CE after 6 h incubation. There was no significant difference in the loss of either CE20:4 or CE18:2 in the presence of MDM when $O_2^{\cdot-}$ production was stimulated, but, as expected, CEs in cell-free controls were relatively conserved.

The 2–6 h phase in these experiments is where the MDM-mediated enhancement of LDL oxidation over cell-free controls becomes apparent. It is important to note that, even during this phase, the MDM which were stimulated with PMA are still producing higher amounts of $O_2^{\cdot-}$ compared with non-stimulated MDM, but this does not result in any additional increase in the rate of cell-mediated LDL oxidation. The combined results of three independent experiments shown that there is no significant increase in LDL CE-OOH when MDM are stimulated with PMA compared with non-stimulated MDM after 6 h incubation in Ham's F10, whereas both MDM conditions were associated with significantly higher LDL CE-OOH levels compared with cell-free controls.

After 24 h incubation, the CE-OOH content of LDL is approximately equal in the incubations with MDM (either with or without PMA) and in cell-free controls (Figure 3a). However, measurement of CE depletion shows that the MDM-containing incubations have significantly lower levels of CE remaining compared with the cell-free controls at 24 h (Figure 4). This result is not unexpected, because we know from other studies (results not shown) that formation of LDL CE-OOH (reflecting the propagation phase of LDL lipid oxidation) is proceeding faster than elimination (reflecting a higher rate of termination reactions) in the cell-free incubations at 24 h, whereas in the cell-containing incubations the highest CE-OOH levels are detected at about 18 h. Beyond this point, reactions which lead to net destruction of CE-OOH are predominating, causing the observed decrease in LDL CE-OOH levels. What is unexpected, however, is the apparent conservation of esters in the MDM-containing incubations which have been stimulated with PMA (Figure 4). These results suggest that MDM-modified LDL is actually less oxidized after 24 h if the respiratory-burst oxidase is stimulated. This is consistent with a slight inhibition of murine-macrophage-mediated oxidation of LDL after stimulation with PMA, recently reported by Jessup et al. [29], but is in contrast to previous reports that have assessed human monocyte (or PBMC)-mediated LDL oxidation by using the TBARS assay [23,25]. PMA was also added to cell-free media containing LDL to assess any possible effect on the extraction efficiency of hexane on CE and CE-OOH, and did not influence the recovery of either of these lipids from supernatants (results not shown).

Ham's F10 medium formulation comprises Fe^{2+} and Cu^{2+} at ~ 3 and $\sim 0.01 \mu M$ respectively. To ensure there was no subtle effect of $O_2^{\cdot-}$ on LDL oxidation which was being obscured by these transition metals, parallel experiments were also performed which were identical with the incubations described above, except that RPMI 1640 medium was used as the modifying medium. RPMI 1640 does not contain added Fe^{2+} or Cu^{2+} , and also contains higher concentrations of Phenol Red (which has antioxidant activity) and is therefore less permissive for LDL oxidation [35]. In our hands RPMI 1640 did not support MDM-mediated LDL oxidation. This was assessed by a lack of production of CE-OOH after 24 h, regardless of stimulation with PMA (Figure 3b), and by the lack of CE consumption after

Figure 5 TBARS formation at advanced stages of LDL incubation in Ham's F10 and RPMI 1640 media

LDL at $100 \mu g/ml$ was incubated with MDM (■), MDM stimulated to produce $O_2^{\cdot-}$ by addition of PMA at $4 \mu g/ml$ (▨) and in medium alone (□). After 7 h or 24 h, $300 \mu l$ of the LDL-containing supernatant was removed and assayed for TBARS as described in the Materials and methods section. All points are means $\pm$ S.D. of triplicate determinations from one experiment, and are representative of three independent experiments.

24 h (results not shown). Since $O_2^{\cdot-}$ was still produced, these results confirm the lack of impact of $O_2^{\cdot-}$ alone on LDL oxidation.

Assessment of LDL oxidation by TBARS assay

The TBARS assay is a commonly used method for measuring the extent of LDL oxidation in studies *in vitro* of cell-free and cell-mediated modification. This assay was used in the present study primarily to allow comparison of results with previous reports of cell-mediated LDL oxidation. TBA reacts with aldehydic end-products of lipid peroxidation (including MDA) under acidic conditions after heating, to form a pink-coloured adduct which can be detected by fluorescence or absorbance spectroscopy [26,32]. Although this assay is non-specific in terms of discrimination of the products of lipid peroxidation [26], previous studies have shown that $\sim 80\%$ of TBARS generated during Cu^{2+} -mediated oxidation of LDL are low-molecular-mass products not associated with the LDL particle [36].

Determination of TBARS in LDL-containing culture supernatants showed that stimulation of MDM with PMA led to a significant increase in TBARS both after 6–8 h of incubation and after 24 h incubation in Ham's F10 (Figure 5). The same effect was generally true for incubations in RPMI 1640, although the levels were much lower than those in Ham's F10, and in the experiment shown the concentrations of TBARS after 24 h in MDM incubations in RPMI 1640 with and without PMA were not significantly different (Figure 5). These results are not consistent with the more direct h.p.l.c. measurements of LDL oxidation presented above, but do agree with previous reports which have shown an increase in TBARS from LDL-containing culture supernatants after stimulation of monocytes (or PBMC) to produce $O_2^{\cdot-}$ in RPMI 1640 [23,25]. In one experiment in which TBARS were measured after 1 h, levels were all < 1 nmol of MDA equiv./mg of LDL protein, and there were no differences between the PMA-stimulated and non-stimulated cells (results not shown). In another single experiment we also determined the amount of TBARS produced by PMA-stimulated MDM in the absence of LDL, and this was 0.6 and 3.5 nmol of MDA equiv./10 ml of RPMI 1640 and Ham's F10 respectively

Table 1 Inhibition of LDL CE depletion and TBARS generation by addition of SOD

Bovine Cu/Zn SOD (10 µg/ml) was added to Ham's F10 containing LDL at 100 µg/ml, then incubated in the absence (cell-free) or presence of cells (MDM) or cells stimulated with PMA at a concentration of 4 µg/ml (MDM + PMA). After 24 h incubation, LDL oxidation was assessed by TBARS and by CE depletion as described in the Materials and methods section.

	Cell-free		MDM		MDM + PMA	
	— SOD	+ SOD	— SOD	+ SOD	— SOD	+ SOD
CE 20:4 (µM)*	7.8	10.4	0.3	4.2	0.7	3.2
Inhibition of depletion (%)†		19 (15–22, <i>n</i> = 2)		26 (22–30, <i>n</i> = 2)		19 (5–27, <i>n</i> = 3)
CE 18:2 (µM)*	120.6	127.4	25.7	66.8	31.2	78.8
Inhibition of depletion (%)†		5 (2–7, <i>n</i> = 2)		32 (20–55, <i>n</i> = 3)		37 (24–58, <i>n</i> = 3)
TBARS‡		23 (8–37, <i>n</i> = 2)		43 (32–53, <i>n</i> = 2)		36 (22–49, <i>n</i> = 2)

* Results for CE depletion are the mean concentration of CE remaining after 24 h incubation in Ham's F10 in each of the conditions (cell-free, MDM or MDM + PMA) in both the absence and presence of SOD.

† Values for the inhibition of CE depletion by SOD are expressed as the percentage of the original (non-oxidized LDL) CE concentration which is protected from oxidation due to the presence of SOD. Typical concentrations of CE in non-oxidized LDL are ~ 13 µM and ~ 132 µM for CE20:4 and CE18:2 respectively. Range and numbers of independent experiments performed in triplicate are shown in parentheses.

‡ Values are the mean percentage inhibition of TBARS formation compared with incubations which do not contain SOD. Typical TBARS values for the various conditions are presented in Figure 5 (ranges and numbers of independent experiments, each performed in triplicate, are in parentheses).

(equivalent to nmol of MDA equiv./mg of LDL, in experiments where LDL was present), suggesting that in some circumstances TBARS do not reflect LDL oxidation.

Effect of SOD on MDM-mediated LDL oxidation in Ham's F10 medium

The spontaneous dismutation of $O_2^{\cdot -}$ produced under these cell culture conditions has a second-order rate constant of $5 \times 10^5 \text{ M}^{-1} \cdot \text{s}^{-1}$, which can be increased approx. 3000-fold by addition of SOD to the culture medium ([37]; rate constant with SOD $1.6 \times 10^9 \text{ M}^{-1} \cdot \text{s}^{-1}$). It might be predicted that, if $O_2^{\cdot -}$ was largely responsible for the MDM-mediated oxidation which was observed, the addition of SOD would prevent MDM-mediated LDL oxidation. However, this prediction was not supported by our data. In the presence of SOD (10 µg/ml is equivalent to dismutation of 60 nmol of $O_2^{\cdot -}$ /min), MDM-mediated LDL oxidation was significantly greater than cell-free control values (as judged by CE depletion and TBARS) in all cases after 24 h incubation. Table 1 shows that the presence of SOD did consistently inhibit this MDM-mediated oxidation to varying degrees, but this inhibition was also observed in cell-free controls and was not dependent on PMA stimulation of respiratory-burst activity. These results confirm our previously published work on the inhibitory actions of SOD in murine-macrophage-mediated LDL oxidation [29]. The data for TBARS presented in Table 1 suggest that, although the overall levels of oxidation are lower in the cell-free conditions, the relative protection afforded by SOD is similar when cell-free controls are compared with MDM conditions. It is more difficult to make the same comparison for the consumption of CE in cell-free versus MDM conditions, because of the relatively limited consumption of CE in the cell-free conditions. However, the data do show that the relative protection afforded by SOD when MDM are compared with PMA-stimulated MDM conditions is approximately the same, i.e. SOD does not have greater protective efficacy (in terms of antioxidant activity for LDL) when an increased $O_2^{\cdot -}$ flux is generated (Table 1).

The addition of SOD resulted in some inhibition of LDL CE-

OOH formation in cell-free controls after 24 h, but no consistent inhibition of CE-OOH in the MDM incubations at 24 h was observed (results not shown). The latter result may be due to the partial inhibitory effect of SOD on LDL oxidation, causing a delay in the time at which CE-OOH levels reach a peak compared with MDM incubations with no SOD added. The net effect of this decreased rate of LDL oxidation is that in some experiments SOD-containing MDM incubations actually contained higher CE-OOH concentrations compared with MDM incubations. One possible explanation for this result is that the length of the propagation phase of LDL CE oxidation in the presence of SOD was extended, possibly due to its chelation of transition metals in the Ham's F10 medium [28,29]. This hypothesis was supported by measurement of CE depletion, which was always less in the SOD-containing incubations (Table 1). Measurement of LDL CE-OOH at late stages of LDL oxidation is therefore not a reliable comparative index of the extent of LDL oxidation under these conditions. The presence of SOD did not significantly inhibit CE-OOH formation in any of the culture conditions at 1 h (results not shown).

DISCUSSION

These studies *in vitro* have focused on the contribution of human MDM $O_2^{\cdot -}$ production as a mediator of LDL oxidation. We have taken care to use LDL which contains very low levels of CE-OOH, and have measured this in all experiments. We have also used a monocyte-derived cell model which is free of platelets and lymphocytes, which may obscure the direct effect of MDM [11,38,39]. In addition, we have used a sensitive h.p.l.c. method to measure LDL CE-OOH specifically at early stages of LDL oxidation. This is the first detailed study of the early stages of human MDM-mediated LDL oxidation using such sensitive and direct techniques, and, to our knowledge, this is the first report to detail the extent of MDM-mediated LDL oxidation during the respiratory burst. These studies demonstrate that human MDM do not enhance the rate of LDL oxidation for at least the first 2 h of incubation. The reasons for the delayed effect of MDM are not clear. It may be that cells are only effective at later stages,

when components of the medium which are vital for LDL oxidation (e.g. reduced metals) becoming limiting (but are largely replenished when cells are present). Alternatively, it is possible that the cells are pro-oxidant, but the antioxidant defences of LDL are not breached and the net increment of cell-mediated oxidation of LDL is therefore still very low.

We also show that, when MDM are stimulated to produce $O_2^{\cdot-}$ at levels approx. 20-fold higher than for non-stimulated cells, this has no impact on LDL CE-OOH formation in Ham's F10 medium during similar incubations. These observations provide evidence against a pro-oxidant role for $O_2^{\cdot-}$ at the early stages of cell-mediated LDL oxidation and are consistent with previously published work showing that LDL subjected to high fluxes of $O_2^{\cdot-}$ produced by γ -irradiation [22] or xanthine/xanthine oxidase ([12]; W. Jessup and S. Gieseg, unpublished work) does not result in substantial oxidation of LDL. However, it has been recently shown that addition of $10 \mu\text{M}$ Fe^{2+} to the xanthine/xanthine oxidase system in the presence of LDL may result in LDL oxidation [41].

The results presented here also show that, even after 24 h incubation of LDL with MDM, there is no difference in the CE-OOH levels in LDL and no additional loss of LDL CE substrates, following MDM stimulation to produce $O_2^{\cdot-}$. The measurements taken at the later time points confirm recent results of Jessup et al. [29], who did not find any difference in the extent of LDL oxidation when mouse peritoneal macrophages were stimulated to produce $O_2^{\cdot-}$, but are in contrast with studies which have assessed human monocyte or PBMC-mediated LDL oxidation by using the TBARS assay [23,25]. However, it is important to note that in both of these previous studies higher levels of $O_2^{\cdot-}$ were produced by the modifying cells, and the initial LDL CE-OOH levels were not reported. This latter point may be important, as it is thought by some that 'seeding' trace amounts of LDL lipid hydroperoxides significantly influences the extent of MDM-mediated LDL oxidation [42], although this issue is still contentious [41].

Under the experimental conditions defined in the present paper, TBARS values do not correlate well with the more sensitive and direct assessment of LDL oxidation (i.e. determination by h.p.l.c. of changes in the content of LDL CE-OOH and non-oxidized neutral lipids). After 24 h incubation, culture supernatants containing LDL and subjected to $O_2^{\cdot-}$ generated through the respiratory burst had significantly higher levels of TBARS compared with non-stimulated conditions which were not reflected by a loss of CE from LDL. Earlier studies using different conditions have similarly provided some evidence for a discordant relationship between LDL oxidation, as assessed by formation of 'high-uptake' LDL, and culture TBARS [23,24,43,44]. Based on the present results and on previous data, we conclude that the TBARS assay is not an accurate method for assessing cell-mediated LDL oxidation.

It is well known that in cell-free systems TBARS related to LDL oxidation correlate well with other measures of oxidation, such as formation of lipid hydroperoxides (at early stages), conjugated-diene formation and formation of fluorescent products [45]. However, in more complex and biologically relevant systems, a number of compounds have been proposed to interfere in the TBARS assay, including proteins, carbohydrates and nucleic acids, although direct evidence for these effects is not strong in all cases [26]. Also, PMA and other pro-inflammatory stimuli can promote the release of arachidonic acid oxygenation products from a number of cells, including murine macrophages, human monocytes and peripheral mononuclear cells [46], which could form TBARS. Although it is clear that the major source of TBARS in cell-mediated LDL oxidation experiments are the

LDL CE, principally CE20:4 and to a lesser extent CE18:2 [47], it is possible that, under conditions where phagocytic cells are stimulated with pro-inflammatory stimuli, non-LDL-derived TBARS could contribute a small but significant amount to the total culture levels.

The reason for the higher recovery of LDL CE from culture supernatants which were previously treated with PMA in some experiments in the present study is unknown. The possibility that PMA directly enhanced the extraction efficiency of hexane for LDL neutral lipids was eliminated by cell-free control extractions. Another possibility was that LDL was being bound to the plasma membrane either by receptor-mediated means or by transient 'complex' formation, and that stimulation with PMA was decreasing this binding. PMA is known to perturb plasma-membrane integrity greatly, and high fluxes of $O_2^{\cdot-}$ have been shown to decrease LDL-receptor-mediated endocytosis, possibly due to changes in plasma-membrane organization [48]. If either of these mechanisms was responsible for the increased yield of CE observed after stimulation with PMA, one would expect a higher recovery of free cholesterol and other less readily oxidized esters such as CE18:1. However, this was not observed, since unesterified cholesterol levels were generally within 10% of each other after 24 h, regardless of stimulation with PMA, and there were no consistent differences in CE18:1 recoveries (B. Garner, unpublished work). It therefore appears that, under these conditions, stimulation of $O_2^{\cdot-}$ production actually decreases the rate of LDL oxidation slightly. It may be argued that, in experiments which use LDL containing only small starting levels of CE-OOH, $O_2^{\cdot-}$ could actually be an electron donor (i.e. reductant) for the one-electron oxidation products of α -tocopherol and/or ubiquinol-10, endogenous LDL antioxidants, thereby inhibiting the chain reactions occurring during LDL lipid-peroxidation reactions ([49]; R. Stocker, personal communication).

The effects of SOD on MDM-mediated LDL oxidation described in this paper are completely consistent with the studies that we recently described in detail (measuring the effects of SOD on murine-peritoneal-macrophage-mediated LDL oxidation, as assessed by iodometrically detectable lipid hydroperoxides and scavenger receptor uptake by macrophages) and hence will not be discussed at length here [29]. The present study shows that addition of SOD to LDL incubations partially inhibited oxidation assessed by TBARS and CE depletion in all cases. Although SOD was used at a concentration which had the capacity to remove more than 450 times the amount of $O_2^{\cdot-}$ generated in these experiments, MDM-mediated oxidation of LDL still proceeded. Several other reports have also shown that SOD is only partially effective in arresting cell-mediated LDL oxidation [12,15,21,24]. This non-specific antioxidant activity may be due to the metal-chelating properties of SOD [28,29].

The evidence that we have presented above strongly suggests that cell-derived $O_2^{\cdot-}$ does not play a rate-limiting role in LDL oxidation. Recent reports have proposed that cells enhance the oxidation of LDL by reducing transition metals present in the medium [19,20]. Since $O_2^{\cdot-}$ has the capacity to reduce transition metals [37], a mechanism could be proposed for $O_2^{\cdot-}$ as a reductant of Cu^{2+} and/or Fe^{3+} and hence indirectly acting as a pro-oxidant. However, under the conditions that we describe, other reductants (e.g. thiols) may compete with $O_2^{\cdot-}$ in reactions with transition metals, and in the presence of cells and sufficient substrates (e.g. cystine), production of these reductants is probably not limited. It has been previously shown that extracellular thiol production is greatly diminished if cystine is not in the medium [20,50]. The physiological relevance of $O_2^{\cdot-}$ mediated metal reduction is unknown, but it should be noted that the

spontaneous dismutation of $O_2^{\cdot-}$ will always be in competition with such reactions, and the available experimental evidence suggests that other cell-derived reductants, such as thiols, may be more important in this scheme [19].

In summary, the present paper shows that: (i) there is a lag period of at least 2 h before MDM enhance the rate of LDL oxidation compared with cell-free controls in Ham's F10 medium; (ii) early stimulation of the respiratory-burst oxidase of human MDM to produce large fluxes of $O_2^{\cdot-}$ does not enhance LDL oxidation in Ham's F10 medium, either during respiratory-burst activity or at later stages; (iii) the TBARS assay does not correlate well with more sensitive and direct h.p.l.c. methods used to measure cell-mediated LDL oxidation and is thus not the method of choice for these types of experiments; and (iv) SOD only partially inhibits MDM-mediated LDL oxidation and also partially inhibits LDL oxidation in cell-free controls. We therefore conclude that it is unlikely that human MDM-derived $O_2^{\cdot-}$ plays a major role in cell-mediated LDL oxidation under the conditions which we have described.

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