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Erratum
Human (THP-1) Macrophages Oxidize LDL by a Thiol-dependent Mechanism†

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The oxidative modification of low-density lipoprotein by macrophages may be an important mechanism in the pathogenesis of atherosclerosis. The human monocytic leukaemia cell line THP-1, when stimulated with phorbol ester, shares many properties with human monocyte-derived macrophages. Oxidation of LDL by these cells was characterised by depletion of α-tocopherol, increases in thiobarbituric acid reactive substances and increases in electrophoretic mobility. The LDL particles were also converted to a form which increased accumulation of cholesteryl esters within macrophages. The oxidative mechanism appeared to be dependent upon the presence of thiols in the cellular medium. Oxidation of LDL by THP-1 macrophages, and production of thiols by these cells, were dependent upon the presence of L-cystine in the medium. Furthermore, cellular oxidation of LDL could be partially mimicked by the addition of cysteine to Hams F10 medium. Macrophage-independent oxidation of LDL, mediated by the addition of copper ions, was inhibited by cystine and cysteine in phosphate buffered saline, but not in Hams F10 medium. The glutathione content of THP-1 macrophages was also dependent upon the presence of cysteine or cystine in the medium, but inhibition of glutathione synthesis by buthionine sulfoximine did not prevent the production of thiols or the oxidation of LDL by THP-1 macrophages.

Key words: Human macrophages, thiols, oxidized low-density lipoprotein, atherosclerosis

Abbreviations: LDL, low-density lipoprotein; EDTA, ethylene diamine tetra-acetic acid; PBS, Dulbecco's phosphate buffered saline; PMA, phorbol 12-myristate 13-acetate; BHT, butylated hydroxy toluene; REM, relative electrophoretic mobility; BSO, buthionine sulfoximine.

INTRODUCTION

Accumulation of lipid-laden foam cells in the subendothelial region of arterial walls is an early event in the development of atherosclerosis.[1] Foam cells originate from monocyte-derived macrophages,[1] which take up modified LDL in an unregulated manner via the scavenger receptor(s).[2,3] Native LDL does not cause foam cell formation because accumulation of cholesteryl esters is prevented by down-regulation of the native LDL receptor.[2] LDL can be modified in a number of ways so that it is recognized by the macrophage scavenger receptor,[4–6] but evidence is

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accumulating that oxidized LDL is the modified form of LDL that contributes to foam cell formation *in vivo*.[3,7,9]

All of the cell types commonly associated with atherosclerotic lesions have been shown to oxidatively modify LDL *in vitro*, including arterial endothelial cells,[10,11] arterial smooth muscle cells,[12] lymphocytes,[13] platelets[14] and macrophages.[15,16,17] A number of mechanisms of cell mediated modification have been proposed, including attack by lipoxygenases[11,15] and/or generation of superoxide.[10,15,18] We have recently shown that modification of LDL by the peroxynitrite anion, which can be formed by the reaction of nitric oxide and superoxide, leads to recognition by the macrophage scavenger receptor,[19] but induction of nitric oxide synthesis in mouse peritoneal macrophages exerted a protective effect upon LDL modification.[20] Similarly, the roles of lipoxygenases and of superoxide generation in LDL oxidation by macrophages have proved controversial.[21–23]

Heinecke *et al.*[24] demonstrated that sulphur-containing amino acids were essential for both superoxide production and oxidation of LDL by monkey arterial smooth muscle cells. More recently, Sparrow and Olszewski[25] reported that oxidation of LDL can occur by cell dependent thiol production in media containing transition metals, possibly by generation of a thiyl radical. It now seems that the oxidation of LDL by different thiols, in the presence of copper ions, may proceed by both superoxide-dependent and -independent mechanisms.[26]

In the present study we have characterised the oxidation of LDL by the human monocytic leukaemia cell line THP-1. These cells are induced, by treatment with phorbol ester, to differentiate into macrophage-like cells[27,28] which mimic human monocyte-derived macrophages in several respects, including induction of scavenger receptors,[29] accumulation of cholesteryl esters[30] and secretion of apoprotein E and lipoprotein lipase.[31] We have examined the role of thiols in this oxidative process, demonstrated the production of thiols by THP-1 macrophages and investigated the interactions between intracellular glutathione content, thiol production and oxidation of LDL.

MATERIALS AND METHODS

Materials

All tissue culture plastics were purchased from Falcon; tissue culture medium, sera and other reagents were purchased from Gibco, BRL. Hams F10 formulated without cysteine/cystine was purchased as a special order from Gibco BRL. Laboratory reagents were purchased from the Sigma Chemical Co. Radiochemicals were purchased from Amersham Life Science U.K. (Bucks.).

Preparation of LDL

Human LDL was prepared by ultracentrifugation in a discontinuous density gradient,[32] and dialysed against PBS containing 100 μM EDTA. The final dialysis medium contained only PBS, in order to allow the cell modification of LDL to be performed under defined conditions. LDL was stored in the dark at 4°C until use or for a maximum of 14 days. These conditions prevented significant changes in oxidizability of the LDL during this storage period. The protein concentration was determined by using the bicinchoninic acid (BCA) protein reagent supplied by Pierce.

Cell Culture

THP-1 macrophages were maintained as described by Hassall.[33] For induction of macrophages, THP-1 cells were seeded at 1×10^6 cells/well into 12-well plates, containing RPMI 1640 medium supplemented with glutamine (4 mM), penicillin/streptomycin (20 IU/ml of each), bovine foetal calf serum (10%, v/v) and PMA (100 ng/ml). PMA was dissolved in DMSO (0.01% final concentration). Cells were maintained for seven days; media containing PMA were replaced at three-day intervals.

Modification of LDL by Macrophages

THP-1 macrophages were washed in Hams F-10 medium before incubation in modification medium, which was defined as Hams F-10 medium containing glutamine (4 mM), penicillin/streptomycin (2IU/ml), supplemented with 3 μM $FeSO_4$, 10 μM EDTA and 100 μg/ml LDL (final vol. 1 ml). The additions of $FeSO_4$ and EDTA to Hams F-10 medium have previously been defined as optimal for the modification of LDL by mouse peritoneal macrophages.[34] In experiments designed to investigate the role of thiols in oxidation of LDL, the Hams F10 was formulated without cystine or cysteine (referred to in the text as Hams F10 without cystine). As cell-free controls, 12-well plates were incubated in modification medium containing the same additions. After incubation at 37°C for the appropriate period of time (usually 18–24 h), the medium was removed from the plates and centrifuged to remove any cell debris (10,000 g, 5 min). BHT was added to the supernatants at a final concentration of 50 μM, in order to prevent any further oxidation occurring. Electrophoretic mobility of LDL samples was measured on agarose gels using the lipoprotein electrophoresis system supplied by the Beckman company.

Measurement of α-tocopherol

Samples (400 μl) of modification medium containing LDL (100 μg/ml) were extracted with *n*-heptane and their α-tocopherol content measured by HPLC exactly as described by Jessup *et al.*[35]

Measurement of Cholesteryl Ester Formation by THP-1 Macrophages

Samples of cell modified LDL, from "donor" THP-1 macrophages (or from corresponding cell-free controls) were diluted in RPMI medium containing BSA (0.2%), glutamine (4 mM), penicillin/streptomycin (20 IU/ml) and BHT (10 μm), to give a final concentration of 15 μg/ml LDL. This medium, containing 10 μM [^{3}H]oleate/BSA complex, was then added to "recipient" THP-1 macrophages, which had been pre-incubated for 9 h in RPMI medium containing 0.2% BSA. Measurement of cholesteryl ester formation was then performed exactly as described.[19] Results are expressed as nmoles [^{3}H]oleate incorporated into cholesteryl ester/mg cell protein.

Measurement of Intracellular Glutathione Content

At the end of the modification experiments, the THP-1 macrophages were washed twice with PBS (1 ml), before lysis in 200 μl of ice-cold 0.1% Triton X-100/PBS, and all lysates were stored on ice until assay. Total glutathione was measured spectrophotometrically as the sum of both its oxidized and reduced forms as described.[36] Results were expressed as nmol of glutathione/mg cell protein, and triplicate wells were assayed for each condition. Buthionine sulfoximine (BSO), when present, was used at a concentration of 100 μM.

Measurement of Thiol Production by THP-1 Macrophages

Thiol concentration was measured exactly as described,[25] using the thiol-specific reagent 5,5'-dithio-*bis*(2-nitrobenzoic acid) (DTNB). In order to minimize interference from additions to the medium, Hams F10 medium alone, formulated with and without L-cystine was used.

RESULTS

Oxidation of LDL by THP-1 Macrophages

The oxidative modification of LDL is characterized by an increase in negative charge of the LDL particle and results in an increase in electrophoretic mobility on agarose gels,[37] which can be expressed relative to the mobility of native LDL and is therefore termed the Relative Electrophoretic

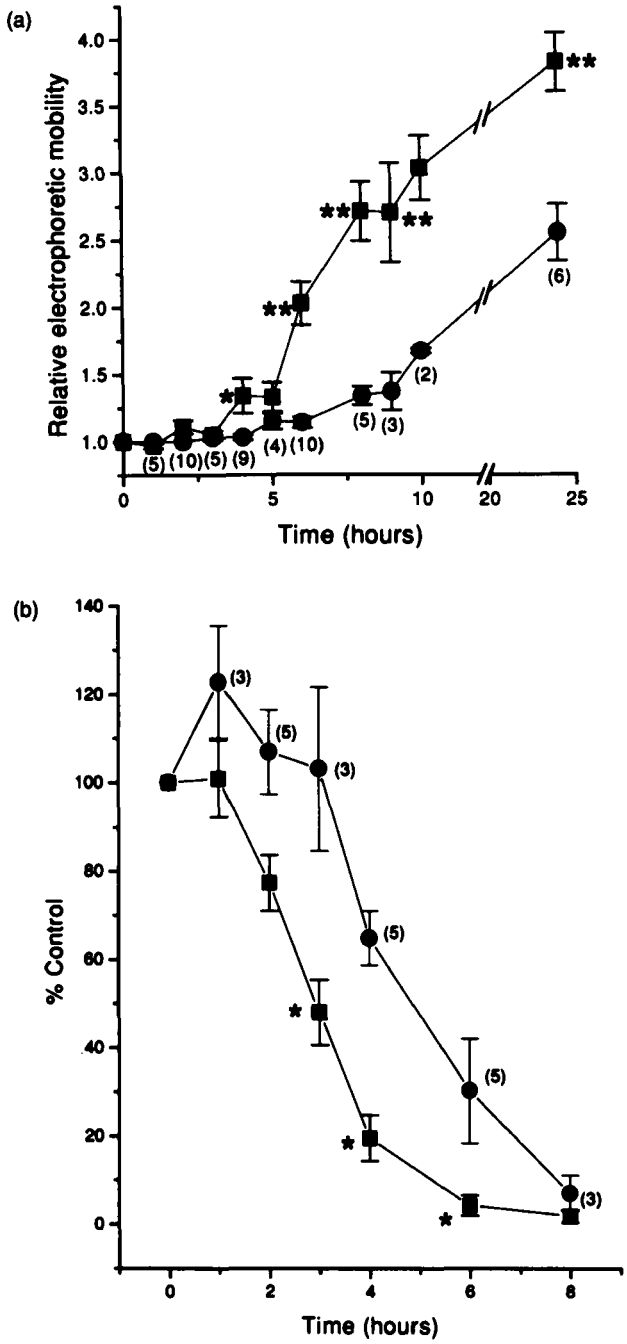

FIGURE 1 Effect of THP-1 macrophages on oxidation of LDL, as measured by depletion of α-tocopherol and increases in relative electrophoretic mobility. THP-1 macrophages (■), or cell-free controls (●), were incubated in the presence of standard Hams F10 modification medium, containing LDL (100 μg/ml) for the periods of time indicated, at 37°C. The medium was then harvested from the cells, or from the corresponding cell-free incubation, and the relative electrophoretic mobility (a) and α-tocopherol content (b) determined as described in the Methods, for the numbers of independent experiments indicated in parentheses. Electrophoretic mobility of modified LDL was assessed, and is expressed, relative to that of native LDL. The α-tocopherol content is expressed relative to the control value at the beginning of the experiment (16.48 ± 3.28 nmol α-tocopherol/mg LDL protein, mean ± S.E.M., n = 5). Values significantly different from the corresponding cell-free control are indicated: *$p < 0.05$; **$p < 0.01$.

Mobility (REM). Modification of LDL by THP-1 macrophages was a time- dependent process, associated with increases in electrophoretic mobility (Figure 1a) and depletion of endogenous α-tocopherol (Figure 1b). A lag phase of around 4 to 5 hours was observed, before significant increases in mobility occurred. During this period, α-tocopherol was consumed in both the cell and cell-free modified LDL; however, in the presence of macrophages, α-tocopherol was completely consumed 2 h before the corresponding cell-free controls (Figure 1b). The initial α- tocopherol content of the LDL used for these experiments was 16.48 ±3.28 nmol/mg LDL protein (n=5, mean±S.E.M.; range 10.3–28.6 nmol/mg), which agrees well with published values.[37,38] In the cell modified LDL, the lag phase was followed by a rapid phase of modification which was virtually complete by 10 h of incubation. The electrophoretic mobility of the cell-free controls also increased over this period but the rate and extent of change was considerably less.

The modification of LDL by THP-1 macrophages, as assessed by REM, appeared to be dependent upon cell density/well, in the range 0.05 to 0.5 × 10^6 cells/well (data not shown). At higher concentrations of cells the rate of modification appeared constant, presumably due to the limiting concentration of LDL present. The rate of LDL modification varied between LDL preparations, but the extent of both cell mediated and cell-free modification over 24 h (the longest time period measured) was found to be extremely reproducible; the mean REM value for cell modified LDL was 3.91 ± 0.11 (mean ± SEM) and for cell-free modified LDL was 2.20 ± 0.13 (mean ± SEM) ($p < 0.0005$, n = 24). In all of the experiments performed, increases in the electrophoretic mobility of LDL could be blocked by the presence of the peroxyl radical scavenger, butylated hydroxy toluene (BHT), indicating that the modification was an oxidative process (data not shown). Modification could also be completely prevented by the presence of the iron chelator desferrioxamine, which inhibited oxidation with an I_{50}(concentration giving 50% inhibition of LDL oxidation) of 2.68 ± 0.62 μM (mean ± S.E.M., n = 3), suggesting a stoichiometric relationship between prevention of oxidation and the content of iron in the Hams F10 modification medium (6 μM).

LDL modified by the presence of THP-1 macrophages also increased the incorporation of [^{3}H]oleate into the cholesteryl ester pool of a second set of "recipient" THP-1 macrophages. In three independent experiments the incorporations of [^{3}H]oleate into cholesteryl ester were 0.385, 0.388, 0.348 nmol/mg (native LDL, 15 μg/ml), 0.333, 0.584, 0.358 nmol/mg (cell-free modified LDL 15 μg/ml) and 0.601, 0.708, 0.433 nmol/mg (cell modified LDL, 15 μg/ml). Results are from 4 wells of THP-1 macrophages in each experiment, and cell modified samples of LDL gave significantly ($p < 0.05$) increased esterification compared with both native LDL and cell-free modified LDL.

Effect of Cystine and Cysteine on Oxidation of LDL, and on Glutathione Content, in THP-1 Macrophages

In order to define the effects of cystine and cysteine, these experiments were performed in Hams F10 medium, which was formulated without cystine. The nonspecific oxidation of LDL was minimised by reducing the incubation period to 18 h. Complete Hams F10 medium contains 200 μM cysteine, which rapidly auto-oxidizes to cystine.[25] In the absence of cystine or cysteine, the oxidation of LDL by THP-1 macrophages was reduced markedly (Figure 2a,b; Tables 1 and 2), and the cellular content of glutathione was markedly depleted (Figure 2c; Tables 1 and 2). Addition of cysteine to Hams F10 medium gave increases in LDL oxidation in both the cell and cell-free incubations, indicating that this thiol was capable of generating LDL oxidation in the absence of cells (Table 1). The addition of L-cysteine also resulted in increases in the glutathione content of the THP-1 macrophages, which were significant at 100 μM cysteine.

TABLE 1 Effect of L-cysteine on LDL oxidation, and on glutathione content, in THP-1 macrophages. THP-1 macrophages were cultured as described in the Methods for seven days. The cell were incubated in standard modification conditions (Methods), in Hams F10 medium, formulated without cystine and containing the additions described below. After incubation for 18 h, at 37°C, the medium was harvested, and the electrophoretic mobility of the LDL assessed on agarose gels. The cells were washed with ice-cold PBS, and their glutathione content determined as described in the Methods, and expressed as nmoles total glutathione/mg cell protein. Results are the means ± S.E.M. for the number of independent experiments shown in parentheses

Additions	Cells		Cell-free
	R.E.M. (LDL)	Glutathione nmol/mg	R.E.M. (LDL)
None	1.69 ± 0.10 (5)	7.53 ± 2.52 (3)	1.28 ± 0.06 (5)[a]
30 μM L-cysteine	1.84 ± 0.10 (5)	26.24 ± 8.42 (3)	1.40 ± 0.11 (5)[a]
100 μM L-cysteine	2.11 ± 0.15 (5)[b]	29.33 ± 7.60 (3)[b]	1.55 ± 0.22 (5)[a]
500 μM L-cysteine	2.49 ± 0.31 (5)[b]	37.21 ± 5.64 (3)[b]	2.31 ± 0.42 (5)[c]

[a] indicates values which are significantly different ($p < 0.05$) from the corresponding incubation in the presence of cells.
[b] indicates values significantly different ($p < 0.05$) from the incubation with cells where not additions were made.
[c] indicates values significantly different ($p < 0.05$) from the cell-free incubation where no additions were made.

The addition of L-cystine to Hams F10 medium resulted in increases in LDL oxidation by THP-1 macrophages, but did not affect oxidation of LDL in the cell-free controls (Figure 2a,b). The addition of cystine resulted in increases in electrophoretic mobility on agarose gels (Figure 2a) and in the thiobarbituric acid reactive substances in the lipid moiety of LDL (Figure 2b). Oxidation was significantly enhanced at 30 μM L-cystine, which agrees well with the reported K_m for L-cystine transport.[39] This dependence of cellular LDL oxidation upon L-cystine has previously been reported for endothelial cells[25] and for smooth muscle cells.[24] The addition of L-cystine also increased the intracellular glutathione content of THP-1 macrophages, with significant increases in glutathione content at around 100 μM L-cystine. There appeared to be an association between the intracellular glutathione content and the ability of THP-1 macrophages to oxidize LDL; we established a linear correlation between cellular LDL oxidation in the presence of cystine (as measured by R.E.M.) and glutathione content (R = 0.898).

TABLE 2 Effect of buthionine sulfoximine on oxidation of LDL, and on glutathione content, in THP-1 macrophages. THP-1 macrophages were cultured as described in the Methods for seven days. The cells were incubated in standard modification conditions, as described in the Methods, in Hams F10 medium, formulated without cystine and containing the additions shown below. After incubation for 18 h, at 37°C, the medium was harvested and the cells washed with ice-cold PBS. The electrophoretic mobility of the LDL was assessed on agarose gels, and the glutathione content of the cell homogenates (expressed as nmol total glutathione/mg cell protein) determined as described in the Methods. Results are the means ± S.E.M. for the numbers of independent experiments shown in parentheses

Additions	Cells		Cell-free
	R.E.M. (LDL)	Glutathione (nmol/mg)	R.E.M. (LDL)
None	1.60 ± 0.12 (5)	13.71 ± 3.83 (3)	1.29 ± 0.09 (5)[a]
L-cystine 500 μM	2.51 ± 0.23 (5)[b]	51.59 ± 6.69 (3)[b]	1.23 ± 0.03 (5)[a]
BSO 100 μM	1.74 ± 0.20 (4)	16.24 ± 6.68 (3)	1.40 ± 0.10 (4)
BSO 100 μM + L-cystine 500 μM	2.54 ± 0.29 (4)[b]	16.83 ± 6.45 (3)[c]	1.55 ± 0.41 (4)[a]

[a] values significantly different ($p < 0.05$) from the corresponding incubation in the presence of cells.
[b] values significantly different ($p < 0.05$) from the incubation with cells where no additions were made.
[c] values significantly different ($p < 0.05$) from the incubation with cells, where L-cystine (500 μM) was added to the medium.

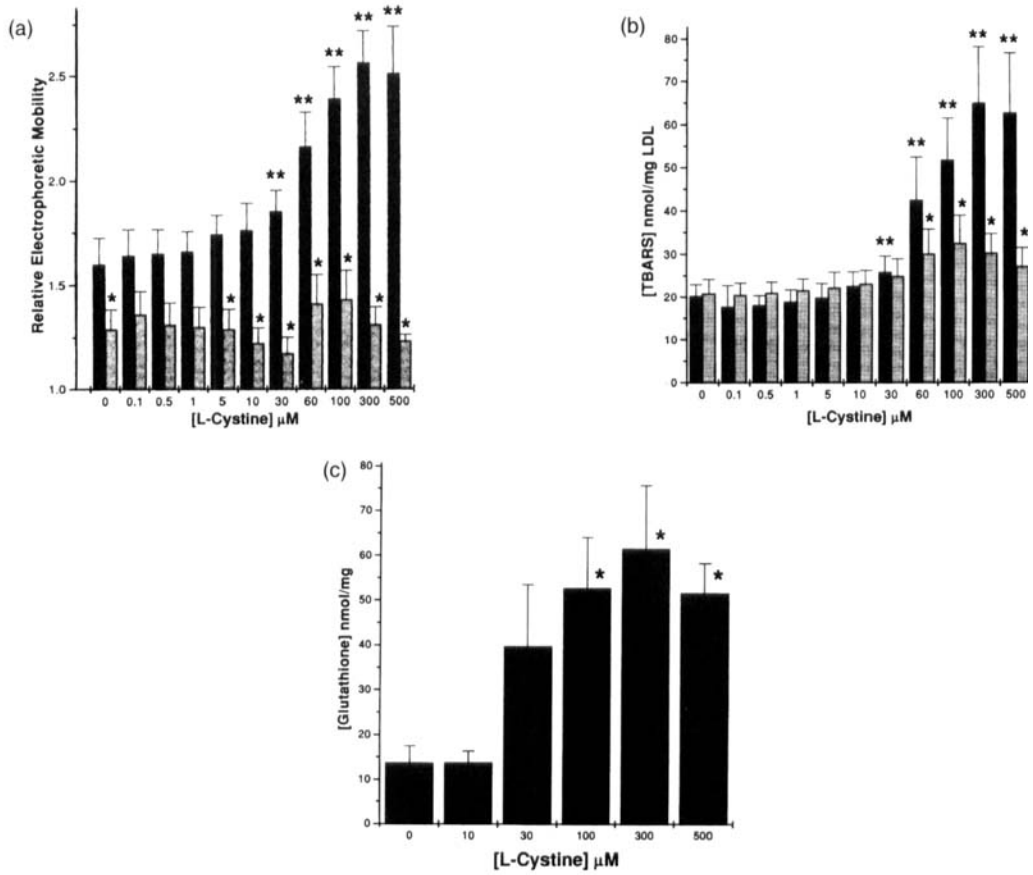

FIGURE 2 Effect of L-cystine on oxidation of LDL, and on glutathione content, in THP-1 macrophage. THP-1 macrophages (■), or cell-free controls (□), were incubated in the presence of Hams F10 modification medium, formulated without cystine, containing LDL (100 μg/ml) for 18 h at 37°C. The medium was then harvested from the cells, or from the corresponding cell-free incubation, and (a) the relative electrophoretic mobility and (b) the thiobarbituric acid reactive substances (TBARS) determined. The cells were washed with ice-cold PBS, and (c) their glutathione content determined as described in the Methods. In Figures 2a and 2b, electrophoretic mobility is expressed relative to native LDL; TBARS are expressed as nmol TBARS/mg LDL protein; and values are the means ± S.E.M. of at least five independent experiments. Values significantly different ($p < 0.05$) from the control incubation where no cystine was added to the cells are indicated**; values significantly different ($p < 0.05$) from their corresponding cell-free controls are indicated*. In Figure 2c, glutathione content is expressed as nmol total glutathione/mg cell protein and values are the means ± S.E.M. of three independent experiments. Values significantly different ($p < 0.05$) from the control incubation are indicated*.

Effect of Buthionine Sulfoximine on Oxidation of LDL, and on Glutathione Content in THP-1 Macrophages

Since this apparent correlation existed between LDL oxidation and glutathione content, we examined the effect upon LDL oxidation of the glutathione synthesis inhibitor, buthionine sulfoximine (BSO).[40] This compound specifically inhibits γ-glutamylcysteine synthetase, and we have previously shown that BSO depletes cellular glutathione levels in THP-1 macrophages, rendering these cells more susceptible to the toxic effects of high concentrations of oxidized LDL.[41] THP-1 macrophages were preincubated for 24 h with 100 μM BSO, before the addition of Hams F10 formulated in the absence of cystine, and supplemented with either 500 μM L-cystine, 100 μM BSO, or L-cystine (500 μM) and BSO (100 μM) together. In the absence of L-cystine, BSO did not reduce the intracellular content of glutathione further (Table 2), and had no significant effect upon LDL

oxidation. In the presence of L-cystine, BSO reduced the intracellular content of glutathione by 67.4%, but did not affect the oxidation of LDL. In a separate series of experiments (n = 3, data not shown), higher concentrations of BSO were used (200–500 μM). No significant decrease in LDL oxidation was observed even at the highest concentrations of BSO employed; in some experiments BSO exerted a slightly pro-oxidant effect. The decrease in glutathione content observed in the presence of BSO does not therefore appear sufficient to prevent oxidation of LDL.

Effect of Cystine and Cysteine on Oxidation of LDL by Copper Ions

Oxidation of LDL can be mediated solely by the addition of high levels of transition metals, such as copper.[37,38] We have used this system to investigate the macrophage-independent effects of cystine and cysteine upon LDL oxidation. The effect of 200 μM cystine, or 200 μM cysteine, on copper oxidation LDL was highly dependent upon the medium to which the copper ions (50 μM) were added. In PBS, cysteine exerted a profoundly antioxidant effect upon the increases in REM (Figure 3), whereas cystine was only marginally antioxidant. In Hams F10 (formulated without cystine), the addition of cystine or cysteine did not significantly affect the oxidation of LDL by copper ions.

Effect of L-cystine, D-cystine and Buthionine Sulfoximine Upon Production of Thiol by THP-1 Macrophages

Rabbit endothelial cells and mouse peritoneal macrophages produce thiol into Hams F10 medium, in a manner which is dependent upon the presence of L-cystine.[25] We examined the time course of production of thiol by THP-1 macrophages in complete Hams F10 medium and in Hams F10 medium formulated without cystine (Figure 4). Production of thiol was essentially linear over the 6 h incubation, in complete Hams F10

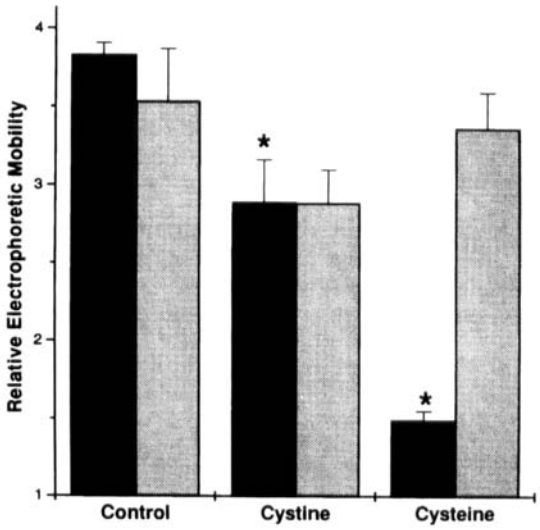

FIGURE 3 Effect of L-cystine and L-cysteine on copper oxidation of LDL. LDL (100 μg/ml) was incubated in either Hams F10 medium (□) (formulated without cystine) or in PBS (■), containing 50 μM $CuSO_4$ and in the presence or absence of 200 μM L-cystine or 200 μM L-cysteine. After incubation for 18 h at 37°C, the electrophoretic mobility of each sample was determined. Results are the means ± S.E.M. of four independent experiments and values significantly different ($p < 0.05$) from the control incubation are indicated*.

medium. Negligible thiol production was apparent in the F10 medium formulated without cystine. As indicated in Figure 4, the production of thiol from THP-1 macrophages in complete Hams F10 medium was variable (range 6.5–37.2 μM). This variability could possibly be due to differences in either the intra-cellular content of L-cysteine at the beginning of the experiments, or in the content of L-cystine in complete Hams F10 medium. We then examined the effect upon thiol production of Hams F10 with and without L-cystine, D-cystine and BSO (100 μM). BSO (100 μM) did not decrease the secretion of thiol by THP-1 macrophages; indeed a slight increase in thiol production was observed (Table 3). L-cystine, when added to Hams F10 medium formulated without cystine, was able to increase thiol production significantly at 500 μM L-cystine. D-cystine did not enhance thiol production by THP-1 macrophages at either 200 μM or 500 μM, confirming that the transport of cystine by the

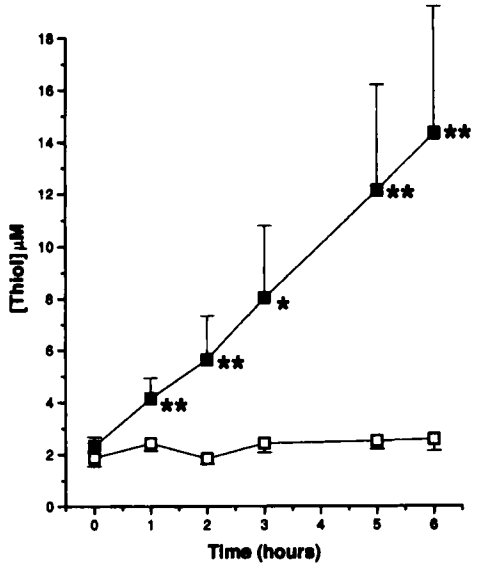

FIGURE 4 Production of thiol by THP-1 macrophages. THP-1 macrophages were cultured as described previously (Methods) before incubation in the presence of Hams F10 formulated with (■) or without (□) cystine. The medium was harvested from the cells after the periods of time indicated, and the thiol content estimated as described in the Methods. Results are expressed as µM thiol, calculated using cysteine as a standard, and are the means ± S.E.M. of six independent experiments. Values significantly different ($p < 0.05$) from the control incubation (0 h) and from the incubation in the absence of cystine are indicated**; values significantly different ($p < 0.05$) only from the incubation in the absence of cystine are indicated*.

THP-1 macrophages is important in the production of thiols.

DISCUSSION

The oxidative modification of LDL by human monocytes/macrophages leads to recognition of the modified LDL particle by the macrophage scavenger receptor.[2,3] The uncontrolled uptake of modified LDL by the scavenger receptor produces enhanced deposition of cholesteryl esters which accumulate in the cytoplasm of the cells, resulting in foam cell formation.[1-3] The accumulation of foam cells appears to be central to the development of fatty lesions within atherosclerotic lesions.[1-3]

We have clearly shown that THP-1 macrophages oxidize LDL in a manner which results in depletion of endogenous antioxidants such as α-tocopherol, increases in electrophoretic mobility and increased production of lipid peroxidation products. This oxidation was dependent upon the presence of transition metals in the Hams F10 medium, as the oxidation could be blocked by the iron chelator desferrioxamine. The dependence of cellular oxidation upon transition metals has been demonstrated repeatedly using differing cell types.[42,43]

The absence of cystine from Hams F10 medium prevented the oxidation of LDL and the production of thiols by these cells, and resulted in depletion of the intracellular glutathione content by around 70–80%. This correlation between the intracellular glutathione content and LDL oxidation suggested that the glutathione content of the cells might be a determining factor in the mechanism of cellular LDL oxidation, possibly via export of glutathione into the culture medium.[40] However, inhibition of glutathione synthesis by BSO indicated that oxidation of LDL can proceed efficiently at relatively low concentrations of intracellular glutathione and that the depletion of glutathione observed in the absence of cystine reflects the absence of intracellular cysteine for the synthesis of glutathione. The reduction in intracellular glutathione by BSO also did not prevent the production of thiol by THP-1 macrophages. Since the intracellular reduction of cystine to cysteine is thought to be achieved at the expense of glutathione,[39] this result indicates either that this process can proceed in the presence of a relatively low level of glutathione, or that an alternative pathway is available to achieve this reduction in these macrophages. It was possible, for example, that the cells secrete reducing equivalents into the culture medium, which could reduce the disulphide to the thiol. However, thiol production was specific for the L-isomer of cystine, suggesting that transport and uptake of cystine into the cells was involved in this process. The direct addition of thiol, as cysteine, to the cell-free incubations, could mimic the increases in electrophoretic mobility generated by incubation in the presence of

TABLE 3 Effect of L-cystine, D-cystine and buthionine sulfoximine on thiol production by THP-1 macrophages. THP-macrophages were cultured as described in the Methods. Cells incubated in the presence of BSO (100 μM) were preincubated for 24 h with the same concentration of BSO. The cells were then incubated in Hams F10 medium as indicated below, without further additions. After incubation for 6 h at 37°C, the medium was harvested, cell debris removed by centrifugation (10 000 g) and the thiol content measured as described in the Methods. Values given by parallel cell-free incubations indicate the background due to the medium and the varying additions. Results are means ± S.E.M. for three independent experiments

Additions	Thiol Concentration μM	
	Cells	Cell-free control
Hams F10 (complete)	8.41 ± 0.80[b]	1.46 ± 0.34[a]
Hams F10 (complete) + 100 μM BSO	12.11 ± 1.28[b]	1.74 ± 0.32[a]
Hams F10 (without cystine)	2.46 ± 0.61	0.84 ± 0.46[a]
Hams F10 (without cystine) + 200 μM L-cystine	10.37 ± 2.79	1.14 ± 0.35[a]
500 μM L-cystine	13.16 ± 1.25[b]	0.75 ± 0.32[a]
200 μM D-cystine	2.43 ± 0.21	0.55 ± 0.23[a]
500 μM D-cystine	3.07 ± 0.56	0.69 ± 0.38[a]
100 μM BSO	1.26 ± 0.62	0.54 ± 0.37

[a] indicates values significantly different from the corresponding incubation in the presence of cells.
[b] indicates values significantly different from the cellular incubation in Hams F10 medium formulated without cystine.

cells. However, much higher levels of exogenous cysteine were required, compared with the level of thiol production by THP-1 macrophages. This may be due to the greater effectiveness of a constant flux of thiol into the medium rather than a single addition of cysteine which presumably would autooxidize rapidly to cystine.[25]

Thiol compounds, oxidizing in the presence of transition metals, may form thiyl radicals ($RS^{\bullet}$), which can then combine with oxygen to thiylperoxy radicals ($RSSO^{\bullet}$), either of which might initiate lipid peroxidation within the LDL[45] particle. Alternatively, the thiyl radical ($RS^{\bullet}$) can react with the thiolate anion to form the disulphide radical anion ($RSSR^{\bullet-}$), which is oxidized by oxygen to yield superoxide and the disulphide.[45,46] Recent work has shown that in a defined buffer system, differing thiols modify LDL by both superoxide-dependent and superoxide-independent mechanisms.[26] The direct extrapolation of this data to the situation in Hams F10 medium must be viewed with caution, however, as we have clearly demonstrated that cystine and cysteine may have widely differing effects depending both upon the oxidizing system and the medium used.

Intracellular thiols, especially glutathione, are considered as antioxidant molecules *in vivo*, which protect biomolecules against free radical damage.[40,45] As discussed above, this may result in the generation of the disulphide (which in the case of glutathione is reduced by the action of glutathione reductase[40]) or the formation of thiyl radicals. Recent work has suggested that thiyl radicals may be removed by the action of the newly characterized thiol-specific antioxidant protein (TSA),[47] found in a wide range of tissues. Extracellularly, however, these protective mechanisms are absent and the production of thiyl radicals, in the presence of transition metals, appears to be one mechanism by which THP-1 macrophages oxidize LDL.

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References

1. R.G. Gerrity (1981) The role of the monocyte in atherogenesis. *American Journal of Pathology*, **103**, 181–190.
2. M.S. Brown and J.L. Goldstein (1983) Lipoprotein metabolism in the macrophage. *Annual Review of Biochemistry*, **52**, 223–261.
3. M.D. Steinberg, S. Parthasarathy, T.E. Carew, J.C. Khoo and J.L. Witztum (1989) Beyond cholesterol. *The New England Journal of Medicine*, **320**, 915–924.

4. J.L. Goldstein, Y.K. Ho, S.K. Basu and M.S. Brown (1979) Binding site on macrophages that mediates uptake and degradation of acetylated low-density lipoprotein, producing massive cholesterol deposition. *The Proceedings of the National Academy of Sciences USA*, **76**, 333–337.
5. R.W. Mahley, T.L. Innerarity, K.H. Weisgraber and S.Y. Oh (1979) Altered metabolism (*in vivo* and *in vitro*) of plasma lipoproteins after selective chemical modification of lysine residues of the apoproteins. *The Journal of Clinical Investigation*, **64**, 743–750.
6. A.M. Fogelman, I. Schechter, J. Seager, M. Hokum, J.S. Child and P.E. Edwards (1980) Malondialdehyde alteration of low-density lipoproteins leads to cholesteryl ester accumulation in human monocyte-macrophages. *The Proceedings of the National Academy of Sciences USA*, **77**, 2214–2218.
7. S. Herttuala, W. Palinski, M.E. Rosenfeld, S. Parthasarathy, T.E. Carew, S. Butler, J.L. Witztum and D. Steinberg (1989) Evidence for the presence of oxidatively modified low-density lipoprotein in atherosclerotic lesions of rabbit and man. *The Journal of Clinical Investigation*, **84**, 1086–1095.
8. U.P. Steinbrecher, H. Zhang and M. Lougheed (1990) Role of oxidatively modified LDL in atherosclerosis. *Free Radical Biology and Medicine*, **9**, 155–168.
9. J.T. Salonen, S. Yla-Herttuala, R. Yamamoto, S. Butler, H. Korpela, R. Salonen, K. Nyyssonen, W. Palinski and J.L. Witztum (1992) Autoantibody against oxidized LDL and progression of carotid atherosclerosis. *The Lancet*, **339**, 883–887.
10. U.P. Steinbrecher (1988) Role of superoxide in endothelial-cell modification of low-density lipoproteins. *Biochimica Biophysics Acta*, **959**, 20–30.
11. S. Parthasarathy, E. Wieland and D. Steinberg (1989) A role for endothelial cell lipoxygenase in the oxidative modification of low-density lipoprotein. *The Proceedings of the National Academy of Sciences USA*, **86**, 1046–1050.
12. J.W. Heinecke, L. Baker, H. Rosen and A. Chait (1986) Superoxide-mediated modification of low-density lipoprotein by arterial smooth muscle cells. *The Journal of Clinical Investigation*, **77**, 757–761.
13. D.J. Lamb, G.M. Wilkins and D.S. Leake (1992) The oxidative modification of low-density lipoprotein by human lymphocytes. *Atherosclerosis*, **92**, 187–192.
14. M. Aviram (1987) Platelet-modified low-density lipoprotein: studies in normals and in patients with homozygous familial hypercholesterolaemia. *Clinical Biochemistry*, **29**, 91–95.
15. A.K. McNally, G.M. Chisolm, D.W. Morel and M.K. Cathcart (1990) Activated human monocytes oxidize low-density lipoprotein by a lipoxygenase-dependent pathway. *Journal of Immunology*, **145**, 254–259.
16. D.S. Leake and S.M. Rankin (1990) The oxidative modification of low-density lipoproteins by macrophages. *The Biochemical Journal*, **270**, 741–748.
17. K.L. Carpenter, J.A. Ballantine, B. Fussell, J.H. Enright and M.J. Mitchinson (1990) Oxidation of cholesteryl linoleate by human monocyte-macrophages in vitro. *Atherosclerosis*, **83**, 217–229.
18. M.K. Cathcart, A.K. McNally, D.W. Morel and G.M. Chisolm (1989) Superoxide participation in human monocyte-mediated oxidation of low-density lipoprotein and conversion of low-density lipoprotein to a cytotoxin. *Journal of Immunology*, **142**, 1963–1969.
19. A. Graham, N. Hogg, B. Kalyanararman, V. O'Leary, V. Darley-Usmar and S. Moncada (1993) Peroxynitrite modification of low-density lipoprotein leads to recognition by the macrophage scavenger receptor. *FEBS Letters*, **330**, 181–185.
20. W. Jessup, D. Mohr, S.P. Gieseg, R.T. Dean and R. Stocker (1992) The participation of nitric oxide in cell free- and its restriction of macrophage-mediated oxidation of low-density lipoprotein. *Biochimica Biophysics Acta*, **1180**, 73–82.
21. W. Jessup, V. Darley-Usmar, V. O'Leary and S. Bedwell (1991) 5-lipoxygenase is not essential in macrophage-mediated oxidation of low-density lipoprotein. *The Biochemical Journal*, **278**, 163–169.
22. C.P. Sparrow and J. Olszewski (1992) Cellular oxidative modification of low-density lipoprotein does not require lipoxygenases. *The Proceedings of the National Academy of Sciences USA*, **89**, 128–131.
23. W. Jessup, J.A. Simpson and R.T. Dean (1993) Does superoxide radical have a role in macrophage-mediated oxidative modification of LDL? *Atherosclerosis*, **99**, 107–120.
24. J.W. Heinecke, H. Rosen, L.A. Suzuki and A. Chait (1987) The role of sulfur-containing amino acids in superoxide production and modification of low density lipoprotein by arterial smooth muscle cells. *The Journal of Biological Chemistry*, **262**, 10098–10103.
25. C.P. Sparrow and J. Olszewski (1993) Cellular oxidation of low-density lipoprotein is caused by thiol production in media containing transition metals. *Journal of Lipid Research*, **34**, 1219–1228.
26. J.W. Heinecke, M. Kawamura, L. Suzuki and A. Chai: (1993) Oxidation of low density lipoprotein by thiols: superoxide-dependent and -independent mechanisms. *Journal of Lipid Research*, **34**, 2051–2061.
27. J. Auwerx (1991) The human leukaemia cell line, THP-1: A multifaceted model for the study of monocyte-macrophage differentiation. *Experientia*, **47**, 22–31.
28. S. Tsuchiya, Y. Kobayashi, Y. Goto, H. Okumura, S. Nakae, T. Konno and K. Tada (1982) Induction of maturation in cultured human monocytic leukaemia cells by a phorbol diester. *Cancer Research*, **42**, 1530–1536.
29. D.P. Via, L. Pons, D.K. Dennison, A.E. Fanslow and F. Bemini (1989) Induction of acetyl-LDL receptor activity by phorbol ester in human monocyte cell line THP-1. *Journal of Lipid Research*, **30**, 1515–1524.
30. C.L. Banka, A.S. Black, C.A. Dyer and L.K. Curtiss (1991) THP-1 cells form foam cells in response to coculture with lipoproteins, but not platelets. *Journal of Lipid Research*, **32**, 35–43.
31. S. Tajima, R. Hayashi, S. Tsuchiya, Y. Miyake and A. Yamamoto (1985) Cells of a human monocytic leukaemia cell line (THP-1) synthesize and secrete apolipoprotein E and lipoprotein lipase. *Biochemical and Biophysical Research Communications*, **126**, 526–531.
32. B.W. Cheung, T. Wilkinson, J.C. Geer and J.P. Segrest (1980) Preparative and quantitative isolation of plasma lipoproteins: Rapid, single discontinuous density gradient ultracentrifugation in a vertical rotor. *Journal of Lipid Research*, **21**, 284–291.
33. D.G. Hassall (1992) Three probe flow cytometry of a human foam-cell forming macrophage. *Cytometry*, **13**, 381–388.
34. D.J. Lamb and D.S. Leake (1992) The effect of EDTA on the oxidation of low-density lipoprotein. *Atherosclerosis*, **94**, 35–42.
35. W. Jessup, S.M. Rankin, C.V. De Whalley, J.R.S. Hoult, J. Scott and D.S. Leake (1990) α-Tocopherol consumption

during low-density lipoprotein oxidation. *The Biochemical Journal*, **265**, 399–405.
36. F. Tietze (1969) Enzymic method for quantitative determination of nanogram amounts of total and oxidized glutathione. *Analytical Biochemistry*, **27**, 502–522.
37. H. Esterbauer, M. Dieber-Rotheneder, G. Waeg, G. Striegl and G. Jurgens (1990) Biochemical, structural and functional properties of oxidized low-density lipoprotein. *Chemical Research in Toxicology*, **3**, 77–92.
38. B. Frei and J.M. Gaziano (1993) Content of antioxidant, preformed lipid hydroperoxides, and cholesterol as predictors of the susceptibility of human LDL to metal ion-dependent and -independent oxidation. *Journal of Lipid Research*, **34**, 2135–2145.
39. S. Bannai (1984) Transport of cystine and cysteine in mammalian cells. *Biochimica et Biophysics Acta*, **779**, 289–306.
40. A. Meister (1988) Glutathione metabolism and its selective modification. *The Journal of Biological Chemistry*, **263**, 17205–17208.
41. N. Gotoh, A. Graham, E. Niki and V.M. Darley-Usmar (1993) Inhibition of glutathione synthesis increases the toxicity of oxidized low-density lipoprotein to human monocytes and macrophages. *The Biochemical Journal*, **296**, 151–154.
42. J.W. Heinecke, H. Rosen and A. Chait (1984) Iron and copper promote modification of low density lipoprotein by human arterial smooth muscle cells in culture. *The Journal of Clinical Investigation*, **74**, 1890–1894.
43. U.P. Steinbrecher, S. Parthasarathy, D.S. Leake, J.L. Witztum and D. Steinberg (1984) Modification of low density lipoprotein by endothelial cells involves lipid peroxidation and degradation of low density lipoprotein phospholipids. *The Proceedings of the National Academy of Sciences USA*, **81**, 3883–3887.
44. O.W. Griffith and A. Meister (1979) Glutathione: interorgan translocation, turnover and metabolism. *The Proceedings of the National Academy of Sciences U.S.A.* **76**, 5606–5610.
45. B. Halliwell and J.M.C. Gutteridge (1989) *Free Radicals in Biology and Medicine*, Clarendon Press, Oxford.
46. H.P. Misra (1974) Generation of superoxide free radical during the autoxidation of thiols. *The Journal of Biological Chemistry*, **249**, 2151–2155.
47. M.B. Yim, H.Z. Chae, S.G. Rhee, P.B. Chock and E.R. Stadtman (1994) On the protective mechanism of the thiol-specific antioxidant enzyme against the oxidative damage of biomolecules. *The Journal of Biological Chemistry*, **269**, 1621–1626.