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Glutathione (GSH) and the Toxicity of Oxidised Low-Density Lipoprotein to Human Monocyte-Macrophages

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Macrophage death, believed to be an important event in the pathogenesis of human atherosclerosis, can be induced by oxidised low-density lipoprotein (LDL) *in vitro*. Supplementation of the culture medium with 5 mM GSH significantly protected human monocyte-macrophages *in vitro* against the toxicity of copper-oxidised LDL.

Oxidation products of LDL include the aldehyde 4-hydroxynonenal (HNE). We present evidence that conjugation of HNE by GSH contributes to this protection. In the absence of cells, HPLC analysis showed there were marked reductions in the levels of both pure HNE and HNE in copper-oxidised LDL in the presence of GSH. However, GSH did not reverse protein modification, as judged by agarose gel electrophoresis, nor did it influence the depletion of polyunsaturated fatty acids, which were assessed using gas chromatography. The possible implications for human atherosclerosis are discussed.

Keywords: GSH, toxicity, 4-hydroxynonenal, oxidised LDL, monocyte-macrophages (human), atherosclerosis

Abbreviations: DNPH, 2,4-dinitrophenylhydrazine; EDTA, ethylenediaminetetraacetic acid; GC, gas chromatography; GSH, reduced glutathione; 7β-OHC, 7β-hydroxycholesterol; HMM, human monocyte-macrophages; HNE, 4-hydroxynonenal; HPLC, high pressure lipid chromatography; LDL, low-density lipoprotein; MPM, mouse peritoneal macrophages; PUFA, polyunsaturated fatty acid; SD, standard deviation; SFM, serum free medium; TLC, thin layer chromatography

INTRODUCTION

The progression of human atherosclerosis from the fatty streak to the advanced lesion is dependent on the formation of an acellular lipid-rich core. An important contribution to core formation and enlargement is believed to be the death of macrophage foam cells.[1] The cause of foam cell death in the lesion is uncertain, but there is *in vitro* evidence that oxidised low-density lipoprotein (LDL) is cytotoxic for mouse peritoneal macrophages (MPM)[2] and human monocyte-macrophages (HMM)[3,4] as well as for other cell types, including smooth muscle and endothelial cells.[5]

The oxidation of LDL produces a complex mixture of lipid hydroperoxides, oxysterols, and aldehydes, all of which are potentially cytotoxic.[6–11]

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The aldehydes arise as breakdown products of lipid peroxides. Peroxidation of the polyunsaturated fatty acids results in the formation of the aldehydes known as hydroxyalkenals, e.g. arachidonate (20:4) yields 4-hydroxynonenal (HNE).[12] HNE has a variety of genotoxic and cytotoxic effects and can, under the appropriate exposure conditions, elicit production of heat-shock proteins,[13] inhibit cell division[14] and induce DNA fragmentation.[15] It is cytotoxic to, and a chemoattractant for, human monocyte-macrophages.[11]

Glutathione (GSH) is a major component of mammalian cellular defences against injury.[16] GSH in association with related enzymes (glutathione peroxidases and glutathione S-transferase) can detoxify hydrogen peroxide and lipid hydroperoxides by catalysing their conversion to water and hydroxy-lipids respectively.[17] GSH can also conjugate with a variety of reactive electrophiles, either spontaneously or catalysed by S-transferase activity.[18] Various thiol-containing compounds, including GSH, conjugate with HNE.[19]

The aim of the present study was to determine whether supplementation of the culture medium with GSH afforded protection for macrophages against the toxicity of oxidised LDL and to detect effects of GSH on HNE levels in incubations without cells.

MATERIALS AND METHODS

Chemicals

Chloroform, ethyl acetate, dichloromethane (analytical Distol grade), and methanol (HPLC grade) were obtained from Fisher Scientific (Leics., UK). [8-^{3}H]-adenine (24 Ci/mmol specific activity) was from Amersham Radiochemicals (Bucks, UK). 24-well culture plates were from Falcon (Becton-Dickinson Labware, Franklin Lakes NJ, USA). Lymphoprep was acquired from Nycomed Pharma AS (Oslo, Norway) and the Macrophage Serum-Free Medium (SFM) from Life Technologies Ltd (Paisley, Scotland, UK). 2,4-Dinitrophenylhydrazine (DNPH) was purchased from Aldrich (Poole, Dorset, UK). Optiphase 'HiSafe' scintillation fluid was from Wallace UK (Milton Keynes, Bucks).

All other chemicals were obtained from the Sigma Chemical Co. Ltd (Poole, Dorset, UK) and were of the highest purity available.

4-Hydroxynonenal-diethylacetal was the kind gift of Dr Georg Waeg, Prof. H. Zöllner and the late Prof. Hermann Esterbauer, of the University of Graz, Austria.

Isolation of Human Monocyte-Macrophages (HMM)

HMM were isolated from peripheral blood as described previously.[3] Essentially, blood was layered onto Lymphoprep. After centrifugation (300*g*) mixed mononuclear cells were removed. Following washing steps to remove platelets, the cells were plated at a density of 3×10^6 cells/ml (1 ml/well) in SFM in 24-well plates. After 1 h, non-adherent cells were removed, leaving adherent HMM.

Cytotoxicity

Cytotoxicity was determined by measuring leakage of radioactivity from cells pre-loaded with tritiated adenine (1 h, 37°C; 0.5 μCi/well), as described previously.[4] After washing with PBS, cells were incubated in SFM in the presence or absence of the various test agents. After appropriate incubation, samples of medium (200 μl) were assessed for radioactivity using a liquid scintillation counter and Optiphase 'HiSafe' scintillant. Intracellular radioactivity was determined by lysing the cells with 1% (v/v) Triton X-100 followed by liquid scintillation counting. Leakage of radioactivity into the medium was calculated as a percentage of the total.

Aldehyde Solutions

HNE was supplied in its diethylacetal form, which was converted immediately prior to use,

to free HNE by acid hydrolysis with 1 mM HCl (1 h, room temperature). The ethanol generated during the hydrolysis was not cytotoxic at the concentrations used.[11] Exact concentrations of the resulting stock solutions (nominally 20 mM) were determined spectrophotometrically at 220 nm, using a molar extinction coefficient of 13 750.

Preparation and Oxidation of LDL

LDL was prepared from normal human serum as described by Havel and co-workers[20] and stored at 4°C with 1 mM EDTA for no longer than one month. Samples were dialysed against PBS to remove EDTA and then oxidised at 1 mg LDL/ml using 5 μM $CuSO_4$ for 24 h at 37°C. Cu^{2+} ions were then removed using Chelex-100 chelating resin. The lipoprotein concentration was determined following filter sterilisation (0.45 μm filter) using the method of Lowry and colleagues.[21] Oxidation of LDL was monitored using gel electrophoresis (Paragon Lipogel System, Beckman UK) and measurement of linoleate, arachidonate and 7β-hydroxycholesterol levels by GC using the method of Carpenter and colleagues,[22] both as described previously.[3] For GC, lipids were extracted, following addition of internal standards (*n*-heptadecanoic acid, coprostane and 5α-cholestane). Bligh and Dyer extraction was followed by reduction with sodium borohydride, saponification and derivatisation.

HNE Analysis

HNE analysis was carried out using an adaptation of the method of Esterbauer and colleagues,[23] as follows. Samples were transferred to 30 ml glass centrifuge tubes, immediately mixed with 4 ml DNPH (1.6 mM in 1 M HCl) and incubated in the dark for 2 h. NaCl (150 mg) was added to each sample. After vortexing, 10 ml chloroform and 1 ml methanol were added, with mixing. The aqueous layer was discarded and the chloroform extract was dried under nitrogen. The residue was dissolved in 2 ml dichloromethane, which was then evaporated under nitrogen and re-dissolved in 0.5 ml dichloromethane prior to TLC. Silica gel-coated glass-backed PK6F TLC plates (20 cm × 20 cm; 1000 μm thickness, Whatman Ltd, Kent) were pre-eluted with ethyl acetate and allowed to air-dry. Samples were applied and the plates eluted in dichloromethane. The appropriate bands, identified by reference to standards, were scraped and the aldehyde–DNPH derivatives recovered using 10 ml dichloromethane per sample. Samples were evaporated under a stream of nitrogen, and re-dissolved in an appropriate volume of methanol. HPLC analysis was then performed using a Hewlett-Packard Series 1050 system fitted with a Spherisorb ODS-2 column (length 25.0 cm, internal diameter. 4.6 mm; PhaseSep, Deeside Clwyd, UK), eluted at 1 ml/min with a linear gradient of methanol in water (77.5% methanol initially, rising to 100% methanol over 20 min). The column was maintained at 40°C. The eluate was monitored at 378 nm, and peak areas measured electronically using a Hewlett-Packard ChemStation data system.

Statistics

Where appropriate, statistical significance was assessed using unpaired Students' t-tests, either alone, or in combination with Analysis of Variance using Microsoft Excel software. Results were considered significant at $P \leq 0.05$.

RESULTS

Protective Effects of GSH against Toxicity

Incubation of HMM for 24 h with 200 μg oxidised LDL/ml resulted in profound radioactivity leakage (ca. 85%; Figure 1A). In these experiments, cells incubated for 24 h with 5 mM GSH alone exhibited a significant increase in leakage compared with the no addition controls (ca. 50%

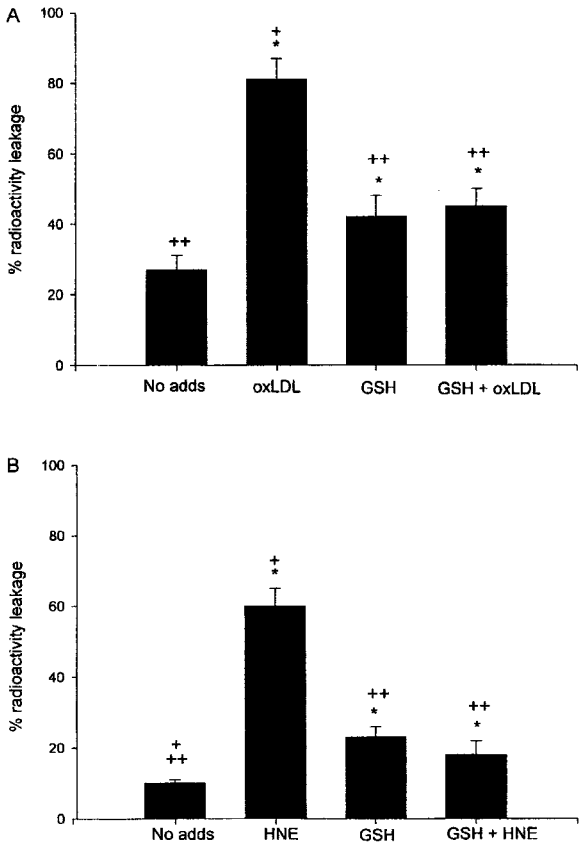

FIGURE 1 *GSH-mediated protection.* All incubations were carried out at 37°C. Toxicity was determined by measuring the leakage of radioactivity from cells pre-loaded with tritiated adenine. The results shown are the mean ± SD from three separate experiments, each carried out in quadruplicate wells. *Indicates significantly different from no additions controls. +Significantly different from GSH control. ++Significantly different from oxidised LDL alone (A), or HNE alone (B). ($P \leq 0.05$). (A) *Oxidised LDL.* HMM were exposed to oxidised LDL (200 μg/ml) in the presence and absence of 5 mM GSH, or incubated as no addition controls, for 24 h. (B) *HNE.* Cells were exposed to pure HNE (200 μM) in the presence or absence of 5 mM GSH, with GSH alone, or incubated as no additions controls, for 6 h.

compared with 30%). In cells co-incubated with GSH and oxidised LDL, the degree of cytotoxicity was not significantly different from the GSH control (ca. 55%). The results indicate that GSH has a significant protective effect against the toxicity of copper ion-oxidised LDL. Lower concentrations of GSH afforded no protection (Table I).

TABLE I Oxidised LDL toxicity for HMM: influence of GSH concentration

Treatment	% Radioactivity leakage
No additions	32 ± 5
oxLDL	86 ± 8*
0.2 mM GSH	38 ± 4†
1 mM GSH	30 ± 6†
5 mM GSH	48 ± 5*†
0.2 mM GSH + oxLDL	88 ± 3*
1 mM GSH + oxLDL	89 ± 4*
5 mM GSH + oxLDL	53 ± 3*†

Cells were incubated for 24 h as no additions controls, with 200 μg oxidised LDL/ml, and with GSH (0.2, 1 and 5 mM) both alone and in combination with oxidised LDL. Results are the mean percentage radioactivity leakage from cells pre-loaded with tritiated adenine and are from three experiments, each carried out in quadruplicate wells ± SD. *Significantly different from no additions controls. †Significantly different from oxidised LDL alone.

HMM exposed to 200 μM HNE exhibited profound increases in leakage of radioactivity (Figure 1B). No additions controls showed modest leakage at 6 h (ca. 10%). HNE produced marked toxicity at 6 h (60% leakage), which was abolished in the presence of GSH. A similar trend was observed at 24 h (data not shown).

HNE Formation and the Influence of GSH

Investigations with samples of free HNE, in the absence of cells, indicated efficient removal of HNE by GSH (Figure 2A) following a 2 h treatment immediately prior to analysis (by HPLC, as the DNPH-derivative). This finding was confirmed, using spectrophotometry, monitoring the free aldehyde at 220 nm. Within 90 min, 5 mM GSH appeared to completely remove 200 μM free HNE (data not shown).

LDL oxidised by copper under the same conditions as those used for the toxicity testing was shown to contain HNE, by HPLC. Treatment of this oxidised LDL at 37°C with GSH resulted in a 58–65% decrease in the concentration of HNE at 2 h (Figure 2B) and 75% at 24 h. This may account, at least in part, for the observed decrease in toxicity of oxidised LDL when incubated with cells in the presence of GSH.

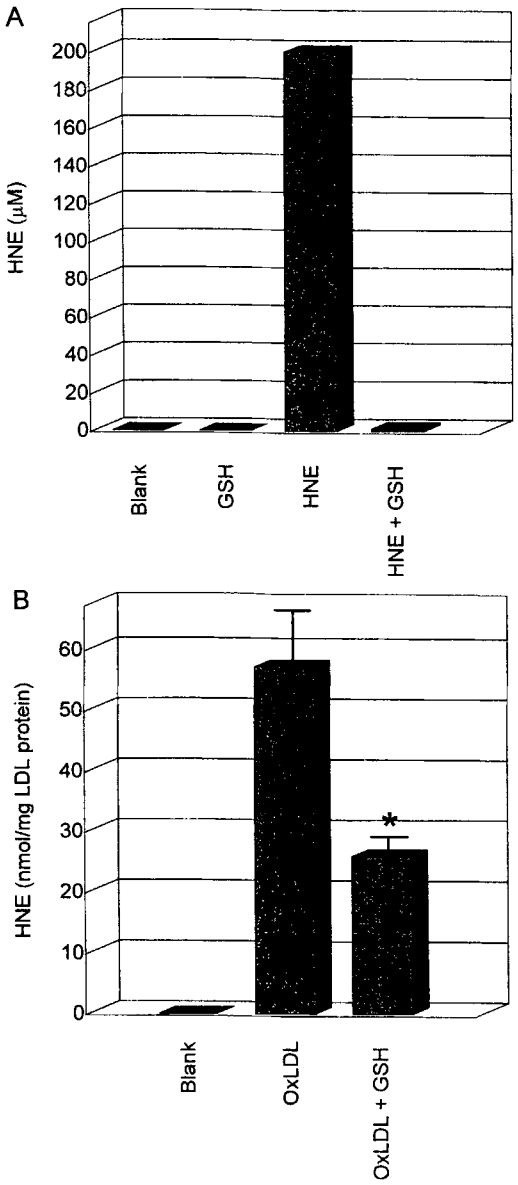

FIGURE 2 *Measurement of HNE levels by HPLC.* All incubations were carried out in the absence of cells at 37°C. HNE was assayed as the DNPH derivative, as described in Materials and Methods. Processed samples (DNPH derivatives) were dissolved in 150 μl (A) or 250 μl (B) methanol, and 20 μl injected onto the HPLC column. (A) *Pure HNE and GSH.* HNE (200 μM) was incubated for 2 h with or without 5 mM GSH. (B) *Depletion of HNE in oxidised LDL by GSH.* Oxidised LDL (900 μg/ml) was incubated for 2 h in the presence or absence of 5 mM GSH. The results shown in (A) are from one experiment, typical of several and are expressed in micromolar concentrations. The results shown in (B) represent the mean free HNE concentrations ± SD of triplicate incubations from one experiment typical of three. *Indicates significantly different from oxidised LDL control $P \leq 0.05$.

REM and Levels of Linoleate, Arachidonate and 7β-OHC

The REM of LDL samples, an indicator of lipoprotein modification, was unaffected by the presence of 5 mM GSH; oxidised LDL incubated for 2 h in the absence of GSH gave an REM of 2.60 ± 0.06 (SD). In the presence of GSH the REM was 2.66 ± 0.06 (SD). These results were from triplicate incubations and the electrophoretic mobilities are expressed relative to native LDL.

Similar results were obtained following incubation for 24 h with GSH (data not shown) suggesting that the lipoprotein modification does not proceed further over the 24 h post-oxidation period.

There was a marked depletion of the PUFAs, as a result of LDL oxidation, with levels falling to around 10 and 4% of initial values, for linoleate and arachidonate respectively (Table II). However, depletion is complete after the 24 h oxidation with copper and it can be seen that further post-oxidation incubation in SFM produced no further depletion, either with or without GSH and in the presence or absence of HMM (Table II). 7β-OHC levels increased with time in the presence of GSH (Table II).

DISCUSSION

The most striking result is the protection conferred against macrophage cytotoxicity of oxidised LDL by supplementation of the culture medium with GSH. It is necessary to distinguish between the effects of GSH in the present study and those obtained previously with metal ion- and cell-mediated oxidation of LDL. Lynch and Frei[24] reported that GSH had pro-oxidant effects on iron-dependent oxidation whilst inhibiting copper-dependent LDL oxidation. However, the protective effects of GSH seen in the present study were not due to inhibition of copper-dependent LDL oxidation, since the LDL used was already oxidised. The protection did not

TABLE II Levels of linoleate, arachidonate and 7β-OHC in oxidised LDL: influence of GSH

Incubation	Time (h)	Linoleate (%)	Arachidonate (%)	7β-OHC (μg/mg LDL protein)
Cells – no GSH	0	9.7	4.0	0.54
Cells – no GSH	2	10.2	4.5	0.19
Cells – no GSH	24	10.1	3.9	0.55
Cells + GSH	0	10.0	4.2	0.10
Cells + GSH	2	9.8	3.6	0.59
Cells + GSH	24	3.4	1.8	2.13
No cells – no GSH	0	8.3	2.0	1.25
No cells – no GSH	2	11.8	6.5	0.95
No cells – no GSH	24	11.4	7.3	1.10
No cells + GSH	0	2.2	1.2	5.51
No cells + GSH	2	2.6	0.3	4.44
No cells + GSH	24	1.5	1.2	9.39

Copper-oxidised LDL prepared as described in Methods, was incubated in SFM, in the presence and absence of HMM, with and without 5 mM GSH. The times indicate the duration of the post-oxidation incubation. Levels of linoleate and arachidonate are expressed as percentages of their levels in native LDL (154.6 and 23.5 μg/mg LDL protein, respectively). 7β-OHC levels in native LDL were 0.5 μg/mg LDL protein. The results shown are from one experiment. Levels of lipids are the total of free plus esterified forms.

appear to be due to a reversal of LDL modification by GSH, because similar relative electrophoretic mobilities were exhibited by copper-oxidised LDL following incubation for 2 h in the presence or absence of 5 mM GSH.

A study of the toxicities of various aldehydic products of lipid oxidation has shown that the most abundant aldehyde in oxidised LDL is hexanal,[25] but this is innocuous.[11] In contrast, the hydroxyalkenals HNE, hydroxyhexenal and hydroxyoctenal were strikingly toxic, with HNE being the most potent.[11] Different hydroxyalkenals are formed from the peroxidation of different polyunsaturated fatty acids (PUFAs). The two most abundant PUFAs in LDL are linoleate and arachidonate, both ω-6 fatty acids, which peroxidise mainly to form hexanal and HNE.[26]

Given the ability of GSH to conjugate with hydroxyalkenals, we investigated the possibility that this is the mechanism of protection against oxidised LDL toxicity. HPLC measurements revealed that HNE levels were 58–75% lower in oxidised LDL when treated with GSH after oxidation (Figure 2D), consistent with removal of HNE by GSH. Nucleophiles such as GSH can attack electrophilic α,β-unsaturated carbonyl compounds, such as HNE, by conjugate addition, termed Michael addition. GSH becomes covalently bound to HNE. This process occurs spontaneously, but more rapidly when catalysed by glutathione transferase.[26] Conjugate addition of GSH to 4-hydroxy-2-alkenals is the first step in the formation of mercapturic acids, a general cellular detoxifying mechanism.[26] Conjugate addition of GSH to HNE is thus a likely explanation for the decrease in HNE levels in oxidised LDL following GSH treatment. The idea that the Michael addition of GSH to HNE is likely to be the crucial step in the removal of HNE is further supported by the observation that levels of hexanal in oxidised LDL did not decrease as a result of the GSH treatment (data not shown). Hexanal has a saturated carbon chain and so cannot participate in conjugate addition.

HNE reacts with various thiols[19] but the Michael reaction is not confined to thiols; for example N_α-acetyl-L-histidine can add HNE.[27] Reaction of proteins with HNE involves Michael addition of protein thiol groups.[28] Also the ε-amino groups of lysyl residues and the imidazole groups of histidine residues in proteins may also undergo Michael additions with HNE.[29,30] By such reactions, together with Schiff-base

modification of lysyl residues, HNE adduction modifies structure and function of cell proteins,[28,31] and such protein changes are believed to contribute to the biological effects of HNE and related aldehydes.[26] For example, HNE-mediated inhibition of the Na^+/K^+-ATPase would compromise ion homeostasis which, if sufficiently severe, would lead to cell death.[31] Maintenance of an adequate tissue GSH status may thus be involved in protecting cells against the adverse effects of HNE *in vivo*, by forming a Michael addition product and preventing HNE modification of cellular proteins.

The most likely mechanism of protection against toxicity of HNE in our view is direct conjugation of HNE with GSH, but we cannot exclude other possibilities such as the augmentation of intracellular defences. Indeed, a protective role for intracellular GSH against oxidised LDL was proposed by Darley-Usmar and colleagues.[32,33] It is possible that in our experiments GSH may be partly taken up by the cells via pinocytosis and act with glutathione peroxidase to detoxify lipid hydroperoxides at or near the cell surface, by converting hydroperoxy-groups to their less toxic hydroxy-analogues. However, cells are generally impermeable to extracellular GSH and supplementation of intracellular GSH requires more complex strategies, including the use of cell-permeable glutathione monoethyl esters, which are converted to GSH inside the cell.[34]

Another possible mechanism of protection by GSH could be by chelation of transition metals. GSH has been reported to be able to form chelation complexes with copper ions[35] and such behaviour may have a protective effect against toxicity. Trace amounts of transition metals may promote decomposition of lipid hydroperoxides, and this may partly be responsible for the toxicity of oxidised LDL. Chelation of transition metals might result in diminished decomposition of these hydroperoxides. However such chelation would be unlikely to account for the reduction in toxicity of pure HNE by GSH, unless this toxicity entails generation of hydroperoxides in the cell surface membrane.

Concentrations of GSH below 5 mM did not protect against oxidised LDL (Table I). This may reflect a limited ability of GSH to enter the lipid phase. Esterbauer and colleagues showed that 77% of the HNE generated in the LDL particle remains associated with the lipid phase.[25] This suggests that a more lipid-soluble thiol compound (such as lipoic acid) might possess enhanced ability to conjugate HNE in oxidised LDL.

A modest but consistent effect was the enhancement of radioactivity leakage in GSH controls as compared with no additions controls. GSH can, under certain conditions, act as a pro-oxidant and we speculate that it is the ability to form the damaging thiyl radical and generate superoxide anion[36,37] which may be responsible for this slight elevation in leakage.

The protection provided by the water-soluble antioxidant GSH is intriguing in being protective against the toxicity of LDL which is already oxidised, a scenario in which α-tocopherol has little effect, and clearly this protection does not involve inhibition of LDL oxidation.

In consideration of the role of antioxidants in atherosclerosis *in vivo* most attention has been paid to the lipid-soluble antioxidants. For instance, Braesen and colleagues reported that probucol inhibits lipid core formation in the WHHL rabbit,[38] thought to be due to inhibition of foam cell death. However, taking into account that GSH can regenerate α-tocopherol levels,[39] in addition to its detoxification activities, it is too simplistic to consider lipid-soluble antioxidants alone. A study of the glutathione status of human atherosclerotic lesions at various stages of development would be of interest.

These results implicate HNE as an important determinant of oxidised LDL toxicity. at least *in vitro*. In the context of atherosclerosis, therefore, the findings suggest a role for thiols in modulating the development and enlargement of the lipid core.

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