Induction of glutathione synthesis by oxidized low-density lipoprotein and 1-palmitoyl-2-arachidonyl phosphatidylcholine: protection against quinone-mediated oxidative stress

Douglas R. MOELLERING*,†, Anna-Liisa LEVONEN‡, Young-Mi GO*, Rakesh P. PATEL†, Dale A. DICKINSON‡, Henry Jay FORMAN†‡ and Victor M. DARLEY-USMAR†‡

*Department of Pathology, Molecular and Cellular Division, School of Public Health, University of Alabama at Birmingham, Volker Hall G019, 1670 University Boulevard, Birmingham, AL 35295-0019, U.S.A., †Center for Free Radical Biology, School of Public Health, University of Alabama at Birmingham, Birmingham, AL, U.S.A., and ‡Department of Environmental Health Sciences, School of Public Health, University of Alabama at Birmingham, Birmingham, AL 35295-0019, U.S.A.

Exposure of endothelial cells to oxidized low-density lipoprotein (oxLDL) leads to diverse cellular effects, including induction of the intracellular antioxidant GSH. It is not known whether lipid- or protein-derived oxidation products cause GSH induction and whether this involves increased activity of the key enzyme in its synthesis, glutamate-cysteine ligase (GCL). Furthermore, the effect of oxLDL exposure on the cell’s ability to combat oxidative stress has not been previously examined. In the present study we found that, in bovine aortic endothelial cells, LDL or 1-palmitoyl-2-arachidonyl phosphatidylcholine oxidized by different reactive oxygen and nitrogen species induced GSH synthesis. However, prevention of GSH synthesis during exposure to oxLDL caused extensive cell death. The mediator causing GSH induction was shown to be a polar lipid and resulted in the increased activity of GCL as well as increased protein levels of the regulatory subunit of GCL. Pretreatment with both oxLDL and the polar lipid subfraction of the oxLDL protected cells against the toxicity of 2,3-dimethoxynaphthoquinone (DMNQ), a superoxide- and H₂O₂-forming compound. The potential of a low level of lipid peroxidation products to initiate cytoprotective pathways is discussed.

Key words: antioxidants, apoptosis, atherosclerosis, glutamate-cysteine ligase.

INTRODUCTION

A wide variety of toxicants are neutralized by the action of the intracellular antioxidant GSH. This versatile tripeptide can be synthesized by the cell, is a scavenger of a number of oxidants and acts as a reducing cofactor for the enzymic detoxification of others [1,2]. However, it is now clear that divergent mechanisms control GSH synthesis in response to toxic or pathological stimuli, including exposure to oxidized low-density lipoprotein (oxLDL) [1–7]. The specific mechanisms for initiators such as oxLDL and the effects of different mechanisms of LDL oxidation on GSH induction are important to understand, given the recent insights that GSH protects the vasculature from reactive oxygen and nitrogen species generated during the atherosclerotic process [8–10].

However, oxidized lipids or lipoproteins can be cytotoxic, with cell death being initiated through both necrotic and apoptotic mechanisms [6,8,11–13]. Relatively little attention has been paid to the effects of exposure of endothelial cells to low concentrations of oxLDL, as may occur in the early stages of a developing lesion. Furthermore, it is now apparent that exercise can induce low levels of lipid peroxidation, and it has been suggested that cell signalling mediated by these non-toxic oxidation products may contribute to vascular protection [14]. The mediators eliciting responses in oxLDL have been partially characterized, and it is thought that oxysterols are responsible for initiating cell death [13]. It is not known whether the molecules that lead to potentially cytoprotective responses are derived from the lipid or protein fraction of oxLDL. Indeed, a number of stable lipid and protein oxidation products, such as 4-hydroxynonenal (HNE) and lipid peroxides, can serve as an early index of exposure to oxidative or nitrosative stress and have less well defined effects on cell function [15].

The pathways responsible for initiating oxidation of lipids in vivo are not completely established. Under conditions of enhanced oxidant stress, increased interaction of superoxide and NO may lead to peroxidation of lipids, including LDL via peroxynitrite as an intermediate [11,16]. Lipoxigenases, cyclooxygenases and peroxidases are also thought to serve as major enzymic catalysts for generating bioactive lipid-oxidation products as signalling molecules at sites of inflammation and vascular disease [8,17]. Particular interest has recently focused upon myeloperoxidase (MPO), a leucocyte-derived haem protein enriched in atherosclerotic lesions. MPO can promote peroxidation.

Abbreviations used: oxLDL, oxidized low-density lipoprotein; nLDL, native LDL; GCL, glutamate–cysteine ligase (γ-glutamylcysteine synthetase, EC 6.3.2.2); PAPC, 1-palmitoyl-2-arachidonyl phosphatidylcholine; DMNQ, 2,3-dimethoxynaphthoquinone; MPO, myeloperoxidase; GCLC, GCL catalytic subunit; SIN-1, 1-morpholinosynergamine; BHT, butylated hydroxytoluene; DTPA, diethylenetriaminepenta-acetic acid; REM, relative electrophoretic mobility; POPC, 1-palmitoyl-2-oleoyl phosphatidylcholine; LOOH, lipid hydroperoxide; BAEC, bovine aortic endothelial cells; GCLM, GCL modifier subunit; lysoPC, lyso phosphatidylcholine; 13-HPODE, (13S)-hydroperoxyoctadecadienoic acid; HNE, 4-hydroxynonenal; EpRE, electrophile-responsive element; HO-1, haem oxygenase-1; PGJ₂, S-series of prostaglandins; LDH, lactate dehydrogenase; LPS, lipopolysaccharide; BSO, buthionine sulfoximine; FOX, ferrous-ion-mediated oxidation of Xylenol Orange.

1 These authors made an equal contribution to this paper.

2 To whom correspondence should be addressed (darley@path.uab.edu).
of LDL lipids through generation of diffusable radical species, including tyrosyl radical [18] and NO-derivated oxidants, such as \( \text{NO}_2 \) [19,20]. During leucocyte activation in serum or plasma, the MPO/\( \text{H}_2\text{O}_2 \) system generates an array of bioactive lipids, including oxysterols and oxidized products of PAPC (1-palmitoyl-2-arachidonyl phosphatidylcholine) [20,21], many of which have been identified as potent signalling species [22]. The physiological relevance of this system is further underscored by the recent demonstration that leucocyte levels of MPO serve as strong and independent predictors of coronary artery disease in subjects undergoing coronary angiography [22a].

It is clear that oxidized lipids derived from these different oxidative processes have the potential to activate a diverse series of signalling pathways in cells [23]. Previous studies have shown that non-toxic concentrations of oxLDL induce the synthesis of GSH in endothelial cells and macrophages [3–5,24]. A strong body of evidence supports the concept that, although production of oxidants is enhanced during the atherosclerotic process, cytoprotective pathways are also induced [24,25]. For example, inhibition of GSH synthesis increases vascular dysfunction in rabbits exposed to a high lipid diet, and GSH protects against apoptosis induced by oxysterols [9,13]. The mechanisms may involve increased activity of the rate-limiting enzyme for GSH synthesis, glutamate-cysteine ligase (GCL; \( \gamma \)-glutamylcysteine synthetase).

Molecular mechanisms leading to adaptive cytoprotection mediated by oxidized lipoproteins and lipids remain poorly defined. With respect to the induction of GSH synthesis, the role of lipid versus protein components of oxidized forms of LDL have not been delineated. In addition, it is not clear whether increased GSH synthesis is due to the increased activity of GCL or other processes such as enhanced cystine transport. Other investigators have implicated a role for the catalytic subunit of Chelex \( ^{\text{TM}} \) (Bio-Rad Laboratories), treated-water, and baked at 500 °C overnight prior to use. Similarly, all the buffers were Chelex*-treated and supplemented with DTPA, as noted. Phospholipids [PAPC or 1-palmitoyl-2-oleoyl phosphatidylcholine (POPC)] from chloroform stocks were divided into portions and dried down under a stream of \( \text{N}_2 \). Lipids were rehydrated (2 mg/ml final conc.) by incubation in polytetrafluoroethylene-lined capped tubes at 37 °C under an argon atmosphere with argon-purged 20 mM sodium phosphate buffer, pH 7.0, supplemented with 200 \( \mu \)M DTPA. The tubes were kept at 37 °C in a water bath for 30–60 min to re-hydrate the lipids, and light was excluded. Rehydration was facilitated by intermittent vortex-mixing. A 1 ml portion of aqueous lipid stock was then transferred to an Avanti Mini-Extruder Set (Avanti Polar Lipids, Inc., Alabaster, AL, U.S.A.) and the lipids extruded ten times through a 0.1 μm-pore-size polycarbonate filter. Vesicles (0.2 mg/ml) were oxidized by incubation with MPO (30 nM), a \( \text{H}_2\text{O}_2 \)-generating system (constant flux of 0.80 μM/min) comprised of glucose (100 \( \mu \)M) and glucose oxidase (100 ng/ml), in the presence or absence of NaNO\(_2\) (0.5 mM) at 37 °C for 12 h. Reactions were stopped by the addition of \( \alpha \)-tocopherol and catalase (300 nM). Lipids from oxidized PAPC or POPC vesicle preparations (1 ml, 0.2 mg/ml) were extracted three times sequentially by the method of Bligh and Dyer [28] immediately after saturating amounts of solid NaCl were added (to enhance lipid extraction). The combined chloroform extracts were evaporated under nitrogen. The vesicles were extracted by adding propan-2-ol,
Measurement of lipid hydroperoxides (LOOHs)

LOOHs were measured by the FOX (ferrous-ion-mediated oxidation of Xylenol Orange) assay [29]. LDL (50–100 µg/ml) was added to the FOX reagent and left in the dark for 30 min at room temperature. The absorbance at 560 nm was then measured versus the appropriate blank. LOOH concentrations were calculated by using an absorption-coefficient \( e_{560} \) value of 43970 M\(^{-1}\) cm\(^{-1}\). The assay was conducted in the presence of BHT. Fresh solutions of the FOX reagent were made prior to every experiment and consisted of Fe\(_{2}\)SO\(_4\) (300 µM), Xylenol Orange (120 µM) and BHT (4 mM) in methanol.

Cell culture

Endothelial cells obtained from bovine thoracic aortas (BAEC) were maintained (37 °C, 5 % CO\(_2\)) in growth medium [Dulbecco’s modified Eagle’s medium containing 1 g/litre glucose (Gibco), 10 % (v/v) fetal-calf serum (Atlanta Biologicals, Norcross, GA, U.S.A.), penicillin and streptomycin]. Cells used in this study were between passages 5 and 10.

Cytotoxicity assays

Cytotoxicity was measured by labelling the cells with annexin V–FITC and propidium iodide (ClonTech) and subsequent FACS analysis [30]. BAEC (5 × 10\(^4\) cells/well) were plated in six-well (35 mm\(^2\)) plates (Costar, Cambridge, MA, U.S.A.) and grown to confluence. The cells were treated with oxidized lipids for 16 h. The cells were then washed twice with PBS and further incubated with 20 µM DMNQ for 24 h. At the end of incubation, the cells were detached using trypsin/EDTA, washed with PBS, and resuspended to 100 µl of annexin V binding buffer, with 0.5 ng of annexin V–FITC and 2.5 ng of propidium iodide. A total of 10\(^5\) cells were analysed on a FACScan (Becton Dickinson, Mountain View, CA, U.S.A.) using WINMDI 2.8 software (The Scripps Research Institute Cytometry Software Page at [http://facs.scripps.edu/software.html]) within 30 min after staining. In some experiments, release of lactate dehydrogenase (LDH) to the medium was used as an index of cytotoxicity.

Measurement of GSH, GCL activity and GCL subunit expression by Western blotting

Total glutathione (GSH + GSSG) was measured using a spectrophotometric assay [31]. The GSH/GSSG ratio and the GCL activity were measured using HPLC methods as described previously [32]. For Western blotting of GCL catalytic (GCLC) and modifier (GCLM) subunits, soluble lysates (30 µg) were resolved by SDS/10 % -(w/v)-PAGE, transferred to a PVDF membrane (Millipore) and probed with antibodies specific to the heavy and light subunits of GCL as described in [6]. Electronic quantification of chemiluminescence was performed on an Alpha-Imager\textsuperscript{TM} from Alpha Innotech Corp., San Leandro, CA, U.S.A.

Measurement of superoxide formation

For measurement of superoxide, coelenterazine (Molecular Probes, Eugene, OR, U.S.A. 10 µM) was added to solutions of xanthine oxidase (0.5–10 munits/ml) and 100 µM xanthine or suspensions of 3 × 10\(^6\) cells/ml in the presence and absence of 5 µM DMNQ. Chemiluminescence was then monitored for 4 min with an Autolumat LB953 luminometer [33]. Superoxide production was determined by using the reduction of cytochrome c in the xanthine oxidase assay to calibrate the chemiluminescence assay.

RESULTS

OxLDL induces GSH synthesis in endothelial cells

Robust induction of GSH synthesis of 4–5-fold occurred on exposure of BAEC to oxLDL over a 24 h period and increased as a function of the concentration of oxLDL (Figure 1). Since several LDL preparations from different plasma donors gave...
similar results, we conclude that the formation of the mediators inducing GSH synthesis were not plasma-donor-specific. A shift in the ratio of reduced (GSH) to oxidized (GSSG) glutathione after induction of GSH by oxLDL would indicate that GSH was decreased by this treatment. To test this hypothesis, GSH and GSSG levels were measured in cells exposed to oxLDL for 16 h. In this experiment the total glutathione increased 2–3-fold, but the ratio of GSH/GSSG was unchanged (47.8 ± 7.6 at 0 h versus 57.2 ± 1.6 at 16 h). The observed values for GSH/GSSG indicate that glutathione was predominantly in its reduced form. Neither nLDL, Cu\(^2+\) or DTPA had a significant effect on total glutathione levels in the cells. No cytotoxicity was detected in the cells incubated with oxLDL at these concentrations as assessed by release of LDH to the medium (Figure 1). It is noteworthy that endothelial cells become much more sensitive to the cytotoxic effects of oxLDL if low serum and subconfluent conditions are used [34,35]. For example, in the present study we found that BAEC treated with oxLDL (150 μg/ml) in medium containing 0.5%, foetal-bovine serum resulted in apoptotic cell death (21% ± 2%) and a minor amount of necrotic cell death (1.5%). All subsequent experiments measuring the effects of ox-LDL on GSH synthesis were performed in 10% (v/v) foetal-bovine serum with confluent cells to model the early stages of an atherosclerotic lesion.

Using oxLDL samples with varying extents of oxidation as reflected in the range of REM values (1.3–2.0), the effects on total glutathione in BAEC was determined (Figure 2). The inset reflected in the range of REM values (1.3–2.0), the effects on atherosclerotic lesion.

Figure 2 Minor oxidation of LDL is required to induce GSH synthesis in BAEC

OxLDL (‘oLDL’) was prepared by incubation of nLDL (2 mg/ml) with Cu\(^2+\) (40 μM) for various periods of time (0–16 h) before the oxidative reaction was stopped with BHT (25 μM) or 50 μM DTPA. Measurement of REM was then performed (inset). Cells were then exposed to 200 μg/ml oxLDL for a period of 24 h before the measurement of total glutathione. Results are means ± S.E.M. for triplicate wells, each containing 10\(^5\) cells exposed to the indicated samples of oxLDL. Abbreviation: cntrl, control.

Table 1 Induction of GSH by various stimuli and peroxide levels within oxLDL

<table>
<thead>
<tr>
<th>Condition</th>
<th>Fold change in [total glutathione]</th>
<th>[Lipid peroxide] (nmol/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1</td>
<td>NA</td>
</tr>
<tr>
<td>Copper-oxidized LDL (150 μg/ml)</td>
<td>3.0 ± 0.75*</td>
<td>118.2 ± 2.2</td>
</tr>
<tr>
<td>nLDL (150 μg/ml)</td>
<td>0.8 ± 0.25</td>
<td>17.9 ± 1.9</td>
</tr>
<tr>
<td>SIN-1-oxidized LDL (150 μg/ml)</td>
<td>3.0 ± 1.0*</td>
<td>73.7 ± 11.8</td>
</tr>
<tr>
<td>SIN-1+ SOD-oxidized LDL (150 μg/ml)</td>
<td>1.4 ± 0.75</td>
<td>135.0 ± 22.2</td>
</tr>
<tr>
<td>Metmyoglobin-oxidized LDL (150 μg/ml)</td>
<td>4.6 ± 1.5*</td>
<td>129.2 ± 1.7</td>
</tr>
<tr>
<td>30 μM Metmyoglobin</td>
<td>0.6 ± 0.25</td>
<td>NA</td>
</tr>
<tr>
<td>LysoPC</td>
<td>25 μM 0.9 ± 0.75</td>
<td>17.9 ± 1.9</td>
</tr>
<tr>
<td></td>
<td>50 μM 1.0 ± 0.75</td>
<td></td>
</tr>
<tr>
<td></td>
<td>100 μM 0.9 ± 0.5</td>
<td></td>
</tr>
<tr>
<td>13-HPODE</td>
<td>20 μM 1.4 ± 0.1*</td>
<td>129.2 ± 1.7</td>
</tr>
<tr>
<td></td>
<td>40 μM 1.63 ± 0.07*</td>
<td></td>
</tr>
<tr>
<td>HNE</td>
<td>10 μM 1.35 ± 0.5</td>
<td>135.0 ± 22.2</td>
</tr>
<tr>
<td></td>
<td>30 μM 3.30 ± 1.25*</td>
<td></td>
</tr>
</tbody>
</table>

Table 1 Induction of GSH by various stimuli and peroxide levels within oxLDL

BAECs were treated with lysopc (25, 50, or 100 μM), HNE (10 or 30 μM), nLDL (150 μg/ml, 16 h, 37 °C), Cu\(^2+\)-oxidized LDL (25 μM Cu\(^2+\), 16 h, 37 °C), SIN-1-oxidized LDL (SIN-1, 2 μM, 6 h, 37 °C), LDL oxidized with SIN-1 (2 mM) + 200 units/ml superoxide dismutase (SOD, 5 h, 37 °C), or LDL oxidized with metmyoglobin (30 μM, 16 h, 37 °C), as well as a metmyoglobin (30 μM) control. LDL was oxidized at a concentration of 1–2 mg/ml. Cells were exposed to these treatments for 16 h and total glutathione was measured. * P < 0.01. Peroxide levels of some forms of oxLDL were also measured by the FOX assay (see the text). Abbreviation: NA, not assessed.
Glutathione induction by oxidized low-density lipoprotein

Figure 3  Induction of GSH by the polar-lipid fraction of oxLDL and oxPAPC

(A) OxLDL was prepared by incubation with Cu^{2+}. OxLDL or nLDL were then extracted into protein and lipid fractions. The cells were exposed to lipid subfraction at concentrations equivalent to 100 μg of LDL protein/ml. GSH was measured 16 h later. Control, no treatment; lipid, lipid extracted from nLDL; nApoB, protein from nLDL; oxlipid, lipid from oxLDL; oxApoB, protein from oxLDL; oxLDL and nLDL have their already defined meanings. (B) oxLDL was prepared by Cu^{2+} oxidation. Lipids were then extracted from oxLDL or nLDL and then partitioned into their neutral and polar fractions and added at equivalent concentrations of 150 μg/ml LDL protein. (C) The cells were exposed to oxPAPC or oxPOPC (100 μg/ml). Total glutathione was measured 16 h later. OxPAPC, Cu^{2+}-oxidized PAPC; MPO oxLDL, MPO-oxidized LDL (extracted lipid fraction, concentration equivalent to that of 100 μg of oxLDL protein/ml); nPAPC, nPAPC; MPO oxPAPC, MPO-oxidized PAPC; nPOPC, native POPC; MPO oxPOPC, MPO-oxidized POPC; veh, vehicle. Results are means ± S.E.M. for triplicate wells, each containing 10^5 cells (*P < 0.01 relative to control).

A component in the lipid polar fraction of oxLDL is responsible for induction of GSH

To determine whether the stimulus for GSH induction is in the lipid or protein fraction of oxLDL, subfractions were prepared and exposed to BAEC, and effects on total glutathione measured (Figure 3A). These data indicate that, while the protein from oxLDL or nLDL had no effect, the lipid fraction of oxLDL increased total glutathione. Lipids extracted from nLDL had no effect on cellular GSH levels, indicating that oxidation reactions, possibly occurring during extraction or solvent addition, did not contribute to the increase in GSH. To determine whether the stimulus is in the neutral or polar fractions, Cu^{2+}-oxidized LDL was prepared and the lipid extracted. A sample was then partitioned into its neutral and polar fractions, and resuspended in 2% (v/v) DMSO and methanol in PBS. Total lipid and polar fractions were added to cells at concentrations equivalent to 150 μg of LDL protein/ml. The neutral fraction was added at an equivalent volume to the polar fraction. In isolated lipid fractions of oxLDL, the polar subtype caused an increase in total GSH (Figure 3B) that was similar in magnitude to that caused by the total lipid extract. Vehicle controls indicated that the solvent mixtures used to re-dissolve the lipid fractions were not responsible for the observed increase in cellular GSH. These results indicate the stimulus for GSH induction is an oxidized polar lipid species. Furthermore, dialysis of oxLDL, to remove low-molecular-mass polar species, abrogated the induction of GSH (result not shown). This supports the conclusion that one or more low-molecular-mass species in the polar lipid fraction was responsible for induction of GSH.

To characterize further the component of the polar lipid fraction responsible for the increase in total glutathione, we exposed BAEC to separate preparations of Cu^{2+}- or MPO-oxidized PAPC, as well MPO-oxidized POPC (100 μg/ml, Figure 3C). Total glutathione was significantly increased by incubation with Cu^{2+}- and MPO-oxidized PAPC, whereas nPAPC or oxPOPC had no effect. Moreover, lipid fractions extracted from MPO-oxidized LDL (equivalent to 100 μg of LDL/ml) also increased total glutathione significantly. These data suggest that oxidized arachidonoyl phosphoholipids are able to induce total glutathione in BAEC.

Inhibition of GSH synthesis enhances the cytotoxicity of high concentrations of oxLDL

We and others have suggested that oxLDL-induced cell death is also related to the GSH status of the cell [3-4, 24, 37]. This is confirmed in Figure 4, in which BAEC were exposed to oxLDL in the absence and presence of buthionine sulfoximine (BSO), the inhibitor of GCL. Cytotoxicity was only evident in cells...
D. R. Moellering and others

Figure 5  GCL activity and GCLM protein is increased on exposure of BAEC to oxLDL

(A) Cells were treated with oxLDL (100 μg/ml, REM = 1.83) for a period of 16 h before preparation of the cell lysate for the measurement of GCL activity by HPLC. The activity of GCL was determined by the formation of γ-glutamyl-α-aminobutyric acid (GABA). Results are means ± S.E.M. for triplicate wells, each containing 10⁵ cells (*P < 0.01 relative to control).

(B and C) Cells were treated with oxLDL (150 μg/ml, REM = 2.1) for 16 h before preparation of cell lysate and subsequent detection of GCLC (B) and GCLM (C) protein by Western blotting. The average chemiluminescence of three control samples was set as 1.0 and the fold increase in oxLDL-treated samples is depicted.

OxLDL increases GCL activity and GCLM protein

In conjunction with GSH, GCL activity in BAEC was increased by oxLDL, whereas nLDL had no effect (Figure 5A). Changes in the levels of GCLC and GCLM in response to oxLDL was assessed by Western blotting. These data (Figures 5B and 5C) demonstrate that oxLDL has no effect on GCLC protein, while increasing GCLM 3-fold.

Preconditioning with oxLDL or oxPAPC protects against quinone-induced oxidative stress

Next it was determined whether pre-treatment with oxLDL can protect endothelial cells against oxidative stress under conditions where GSH synthesis was stimulated. Endothelial cells were pretreated with oxLDL and then the LDL was removed before exposure to DMNQ. To determine whether DMNQ can produce superoxide in endothelial cells, the chemiluminescence from coelenterazine was measured and superoxide production rates calculated by comparison with production from xanthine oxidase and xanthine. In the presence of 5 μM DMNQ, endothelial cells produced 57 ± 5 pM superoxide/min, whereas in control cells without DMNQ treatment the amount produced was 3 pM/min.

Figure 6  Pretreatment of BAEC with oxLDL, oxidized polar lipid or oxPAPC prevents apoptosis induced by DMNQ

(A) Cells were incubated with or without oxLDL (150 μg/ml) or polar-lipid fractions of oxLDL or nLDL for 16 h before the medium was changed and the cells were exposed to DMNQ (20 μM) for 24 h. (B) Cells were incubated with or without ox- or n-PAPC (100 μg/ml) for 16 h before the medium was changed and the cells were exposed to DMNQ (20 μM) for 24 h. All cells were then labelled with annexin V and propidium iodide and analysed with FACSscan. The data show total cell death, including necrotic and apoptotic cell death, expressed as percentage changes over the total cell population. Results are means ± S.E.M., n = 3 (*P < 0.001 relative to its control; #P < 0.01).

To determine the effect of oxLDL on DMNQ-induced cell death, BAEC were examined by FACS analysis using a combination of annexin V and propidium iodide staining. In these experiments, those cells staining positive only with annexin V are designated apoptotic, whereas those only with propidium iodide are assumed to be necrotic. BAECs were treated with DMNQ over a very narrow dose-response curve in which complete cell death was generally evident between 15 and 25 μM. In these experiments a concentration of 20 μM for a period of 24 h was selected and resulted in cell death (mainly apoptotic), which varied between 40 and 100% (Figure 6A). However, pretreatment with either oxidized LDL or the oxidized polar-lipid fraction protected BAECs from DMNQ-induced cell death by approx. 20–25%. There was a small, but significant, effect of polar-lipid fraction extracted from nLDL. Also oxPAPC, but not native PAPC, was able to cause a similar (over 20%) reduction in apoptotic cell death mediated by DMNQ (Figure 6B).

DISCUSSION

The ability of GSH to protect the cell against oxidative stress is generally well accepted. In the present study, the capacity for oxLDL to induce GSH was examined at concentrations of oxLDL that are not cytotoxic. The extent of modification of the LDL particle to achieve this effect was minor and similar to that reported for circulating forms of LDL known as minimally modified forms of oxLDL [8,38]. Despite the low level of modification, robust induction of GSH was observed, depend-
ing on the extent of oxidation, duration of exposure and concentration. As there was no net change in the ratio of GSH to GSSG, the capacity of GSH to act as a reducing cofactor was not impaired after exposure to oxLDL for periods up to 16 h. With respect to enzymes for which GSH is a cofactor, such as glutathione peroxidases or transferases, and non-enzymic conjugation (with HNE for example), the increase in GSH would be expected to have a major impact in enhancing these potentially protective pathways. However, the mechanisms that lead to oxidation of lipids in vivo are largely unknown, and different oxidation mechanisms could yield diverse mixtures of oxidation products. It is clear from the present study that induction of GSH occurred with oxLDL prepared by diverse mechanisms. Metmyoglobin oxidizes LDL in a LOOH-dependent manner, whereas SIN-1 oxidizes LDL in an LOOH-independent manner via formation of the oxidant peroxynitrite [27, 39]. Furthermore, the effect of SIN-1 was substantially inhibited by superoxide dismutase, which has been shown to inhibit peroxynitrite formation from SIN-1. The fact that various methods of LDL oxidation yield similar results in GSH induction is consistent with a secondary product of lipid peroxidation mediating this effect.

An important finding from the present study is that a polylipid component in oxLDL is capable of inducing the synthesis of GSH through a mechanism involving the increased activity of GCL. The increase in GCL activity was of a similar magnitude to that shown in a number of studies using different stimuli such as redox-cycling xenobiotics [6, 40–42]. The two factors that determine the rate of GSH synthesis in the cell are the substrate availability through cystine import, as well as the expression of the heavy and light subunits of GCL. In this respect, previous reports have shown that the cystine transporter is indeed activated by oxLDL [43] and that oxLDL increases the mRNA levels of GCLC. However, in the present study this was not reflected in increased GCLC protein. Instead, a 3-fold up-regulation of GCLM was observed. While GCLC has all the catalytic activity and is feedback-inhibited by GSH, the light subunit has an important regulatory role, since it decreases the $K_i$ for GSH, increasing the catalytic activity [44]. Therefore increases in GSH synthesis may occur without changes in GCLC expression.

At this juncture the mechanism by which GCLM is regulated by oxLDL is not known. Typically, inducers of GCL activity cause either a transcriptional up-regulation of GCLM or GCLC, or both [2]. The promoters of both genes of GCL subunits have electrophile responsive elements (EpREs) that are responsible for the basal GCL transcription of GCL as well as the inducible expression by various oxidants and electrophilic compounds. However, GCLM and GCLC are independently regulated, and there are cell-specific differences in the basal as well as induced expression of GCL subunits. EpRE is also present in the promoter regions of a number of other enzymes involved in detoxification of xenobiotics or antioxidant defence, including glutathione S-transferase, ferritin and haem oxygenase-1 (HO-1) [21, 45]. It is noteworthy that oxPAPC causes an induction of HO-1 in endothelial and smooth-muscle cells [46], presumably via EpRE [47]. It is therefore plausible that the induction of GCLM in BAEC would occur through an EpRE-dependent mechanism.

In the present study we have shown that oxPAPC is able to increase total cellular glutathione. OxPAPC contains several compounds with specific biological activities. While studies have mainly focused on the effects of these compounds on leukocyte adhesion and chemotaxis, they also have cytoprotective properties, such as induction of HO-1 [46]. Intriguingly, the subfraction that is most potent in inducing HO-1 has been found to contain structural analogues of the J-series of prostaglandins (PGJs) [22, 46]. We have recently found that PGJs are able to increase total glutathione and GCLM mRNA at very low concentrations ($\approx 150$ nmol/L) in human endothelial cells [46a]. It is therefore reasonable to postulate that small amounts of oxidized products of arachidonyl phospholipids present in oxLDL contain specific lipid compounds that are able to initiate protective pathways, thereby protecting against the toxicity of oxLDL.

Recently it was suggested a role of lipid hydroperoxides in GSH induction in macrophages, with a minor involvement of protein modification and scavenger-receptor-mediated signalling [4, 44]. These results are largely based on the fact that, when LDL is oxidized in the presence of ebselen, a glutathione peroxidase mimetic, the peroxide content is decreased and GSH induction attenuated. In our study the amount of lipid hydroperoxides present in oxLDL did not correlate with the magnitude of glutathione induction. Moreover, 13-HPODE concentrations at the higher end or above the levels present in oxLDL were not able to increase total glutathione to the same extent as oxLDL (Table 1). It should be noted that lipid hydroperoxides (‘seeding peroxides’) initiate the oxidative modification of phospholipids in the LDL particle, and therefore ebselen would not only decrease lipid hydroperoxide levels, but also oxidized phospholipids. We conclude that lipid hydroperoxides make only a minor contribution to the induction of glutathione in BAEC.

A number of aldehydes are present in oxLDL, including HNE [37]. The effects of HNE on GSH in endothelial cells has not been reported, but it is has been shown to increase GSH synthesis in lung epithelial cells through induction of GCL [6]. HNE has been detected in human atherosclerotic lesions, and the GSH-dependent enzymes for its detoxification are present in models of vascular disease [15, 48]. It is present in oxLDL at concentrations of approx. 5 nmol/mg of LDL or 0.2–1.0 $\mu$mol under the conditions shown in Figure 1 in which increased GSH synthesis by oxLDL was demonstrated [37]. These levels are 20–100 times below the concentration of HNE (10–30 $\mu$mol) necessary to elicit a similar increase in GSH (Table 1).

Superoxide and $H_2O_2$ are thought to contribute to the pathophysiology and toxicity of numerous agents that exert deleterious effects on the vasculature [49]. Measurement of the rate of superoxide production in BAEC on exposure to DMNQ gave values in the order of picomol/min similar to that measured for the activation of NADPH oxidases in endothelial cells [49]. The data presented here suggest that exposure of cells to oxidized lipids can protect against the subsequent exposure to oxidants. The same lipid fraction capable of inducing GSH was also responsible for the protection against the cytotoxicity of DMNQ. The findings are also consistent with a role for GSH in the cytoprotective mechanism, although this could not be tested directly, since inhibition of GSH synthesis with BSO greatly enhanced the cytotoxicity of both oxLDL and DMNQ.

While it is clear that, at higher concentrations, oxLDL can be cytotoxic through mechanisms dependent on oxysterols in the non-polar lipid fraction [15, 50], other signalling pathways are induced in cells by oxLDL that are potentially cytoprotective. These can be clearly separated from the pro-apoptotic effects of oxLDL that occur under the more stressful conditions of low confluency and serum concentration that are no doubt relevant to the more severely compromised vasculature in an advanced atherosclerotic lesion.

In conclusion, we have demonstrated that induction of GSH synthesis by a lipid oxidation product in the polar fraction of oxLDL and in oxPAPC is associated with cytoprotection against oxidants generated from DMNQ. Minor oxidative modification of LDL may occur in a number of physiologically relevant contexts. For example, it has been reported that moderate exercise causes lipid peroxidation, and this is associated with an...
adaptive response to protect the vasculature from oxidative stress [14]. The present study suggests both a mechanism and an approach to identify the lipid mediators that may be involved in this process.

We thank Stanley L. Hazen and David Schmitt (Departments of Cell Biology and Cardiovascular Medicine, Cleveland Clinic Foundation, Cleveland, OH, U.S.A.) for generously providing MPO-oxidized LDL, oxPAPC and oxPOPC. This study was supported by National Institutes of Health grants NIH RO1ES/HL10167 and NIH ES 05511. A.-L.L. is supported by The Academy of Finland, The Finnish Medical Society Duodecim, The Finnish Cultural Foundation and The Foundation for Cardiovascular Research in Finland, R.P.P. is Parker B. Francis Fellow in Pulmonary Research.

REFERENCES


Glutathione induction by oxidized low-density lipoprotein


Received 19 September 2001; accepted 28 November 2001