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

Douglas R. MOELLERING^{*1}, Anna-Liisa LEVONEN^{*1}, Young-Mi GO^{*}, Rakesh P. PATEL^{*†}, Dale A. DICKINSON[‡], Henry Jay FORMAN^{†‡} and Victor M. DARLEY-USMAR^{†‡2}

^{*}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 are 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]. Lipoxygenases, 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, 3-morpholinylsindnonimine; 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, lysophosphatidylcholine; 13-HPODE, (13S)-hydroperoxyoctadecadienoic acid; HNE, 4-hydroxynonenal; EpRE, electrophile-responsive element; HO-1, haem oxygenase-1; PGJs, J-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 diffusible radical species, including tyrosyl radical [18] and NO-derived oxidants, such as NO_2 [19,20]. During leucocyte activation in serum or plasma, the MPO/ H_2O_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; γ -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 GCL (GCLC) in causing an increase in GSH, but this was based upon modest changes in mRNA levels that may not result in significant changes in protein [24]. The present study defines the role of GCL in regulating GSH levels in response to oxLDL. Furthermore, a potential role for several oxidized lipid intermediates has been demonstrated, most notably in the oxPAPC fraction of oxLDL modified by either Cu^{2+} or MPO. The significance of these results for the adaptive cytoprotection of endothelial cells against oxidative stress is demonstrated by exposure to non-toxic levels of oxLDL and measurement of apoptosis induced by superoxide and H_2O_2 , generated by the redox-cycling compound DMNQ.

EXPERIMENTAL

Isolation and oxidation of LDL

Human LDL was isolated from plasma of healthy donors by differential centrifugation using the method previously described [26]. The concentration of LDL protein was determined using the Bradford protein assay reagent (Bio-Rad). All native or oxidized LDL concentrations used in the experiments were normalized to this protein concentration.

For oxidation, LDL (1–2 mg/ml) was incubated with Cu^{2+} , Mb^{3+} and 3-morpholininosynonimine (SIN-1), and aliquots were taken at the specified times. SIN-1 was used to generate equimolar concentrations of superoxide and NO, mimicking the formation of peroxynitrite [27]. Reactions were stopped by addition of butylated hydroxytoluene (BHT; 50 μM) or diethylenetriamine-penta-acetic acid (DTPA; 100 μM). In some incubations with SIN-1, superoxide dismutase (200 units/ml) was also added. The relative electrophoretic mobility (REM) of LDL was determined

on agarose gels using the lipoprotein electrophoresis system supplied by Beckman.

Protein and lipid fractions from native LDL (nLDL) and oxLDL were obtained by chloroform/methanol extraction. Briefly, 1.5 ml of ice-cold PBS plus 100 μl of 100 μM DTPA and 2 ml of methanol were added to 0.5 ml of nLDL or oxLDL (2 mg/ml). The resultant solution was vortex-mixed for 1 min followed by the addition of 4 ml of chloroform and further vortex-mixing for 1 min. The solution was then centrifuged at 2000 g for 5 min at 4 °C. A 3.6 ml portion of the chloroform (bottom) layer was carefully taken and dried under nitrogen. The dried lipid fraction was re-dissolved in 0.9 ml of PBS/DMSO (50:1, v/v) at an equivalent concentration of 2 mg of LDL protein/ml. The protein fraction was collected, washed twice with ice-cold water (2 ml) and once with ice-cold acetone (2 ml). A 0.5 ml portion of *n*-octyl glucoside (30 mg/ml) was added to the mixture and the solution vortex-mixed for 5 min, followed by the addition of 0.5 ml of PBS to give a final concentration of approx. 2 mg/ml.

For fractionation of lipids into polar and neutral fractions, extracted lipids from oxLDL were dried under nitrogen and redissolved in 20 ml of aq. 87% ethanol/hexane (1:1, v/v). Ethanol saturated with respect to hexane, and hexane saturated with respect to ethanol and water were used in these studies. The two layers were collected using a separating funnel and the ethanolic layer washed with hexane (10 ml), and conversely, the hexane layer washed with ethanol (10 ml). The respective solvents were separated again and pooled with earlier fractions, and dried under nitrogen. The ethanolic extract contains the polar-lipid fraction (mainly phospholipids) and hexane contains the neutral lipids (mainly triacylglycerols, cholesterol and cholesteryl esters). Samples were redissolved in 1 $\times$ PBS with methanol/DMSO (1% each, v/v, final concns.) and effects on GSH synthesis in endothelial cells examined.

Preparation and oxidation of phospholipid vesicles by MPO

All glassware was pre-washed with DTPA-containing buffers, rinsed with Chelex® (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 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 μM 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 H_2O_2 -generating system (constant flux of 0.80 $\mu\text{M}/\text{min}$) comprised of glucose (100 μM) 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 α -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,

heptane and 2 M acetic acid (40:10:1, by vol.) and vortex-mixing vigorously. Heptane was added (1:1, v/v) to the above solution, which was then vortex-mixed and then centrifuged 2000 *g*. The upper organic phase was collected and the aqueous phase was re-extracted with heptane three more times. After combining the organic phases, the lipids were dried down under N_2 , and then resuspended in methanol. Samples were then re-extracted using chloroform/methanol extraction prior to addition to cells as described above for the LDL fractionation.

Measurement of lipid hydroperoxides (LOOHs)

LOOHs were measured by the FOX (ferrous-ion-mediated oxidation of Xylenol Orange) assay [29]. LDL (50–100 $\mu\text{g/ml}$) 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 (ϵ_{560}) value of 43970 $\text{M}^{-1}\cdot\text{cm}^{-1}$. 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_2SO_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 (5×10^5 cells/well) were plated in six-well (35 mm^2) plates (Costar, Cambridge, MA, U.S.A.) and grown to confluency. 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[®] 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

Figure 1 Induction of total glutathione in endothelial cells by oxLDL

(A) BAEC were exposed to the concentrations of oxLDL shown for a period of 12 h. The cells were then lysed and the total glutathione content was determined and normalized to cellular protein. (B) Cells were exposed to 250 $\mu\text{g/ml}$ oxLDL for the periods shown and total glutathione was determined. (C) The viability of cells exposed to increasing concentrations of oxLDL (16 h) was determined by the proportion of LDH released into the medium relative to the total LDH in both the cell lysate and medium. The independent, superimposed open square and circle represent the values for nLDL and Cu^{2+} controls respectively. Results are means $\pm$ S.E.M., $n = 3$ or 4. * $P < 0.01$ relative to control.

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 $\mu\text{g}/\text{ml}$ oxLDL for a period of 24 h before the measurement of total glutathione. Results are means $\pm$ S.E.M. for triplicate wells, each containing 10^5 cells exposed to the indicated samples of oxLDL. Abbreviation: ctrl, control.

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 $\mu\text{g}/\text{ml}$) in medium containing 0.5% foetal-bovine serum resulted in apoptotic cell death ($21 \pm 2\%$) 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 to this Figure shows the resulting REM, and the data demonstrate that minor modification of LDL (REM = 1.3–1.5) results in a 3–4-fold increase in total glutathione.

To show that GSH induction was not solely a property of Cu^{2+} oxidation, a number of other methods were used to oxidize LDL (Table 1). Both metmyoglobin- and SIN-1-treated LDL stimulated GSH production in BAEC. Another component of oxLDL that has been implicated as a cell-signalling molecule is lysophosphatidylcholine (lysoPC). However, lysoPC had no effect on GSH induction at concentrations equivalent to what would be

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 $\mu\text{g}/\text{ml}$, 16 h, 37 $^{\circ}\text{C}$), Cu^{2+} -oxidized LDL (25 μM Cu^{2+} , 16 h, 37 $^{\circ}\text{C}$), SIN-1-oxidized LDL (SIN-1, 2 mM, 6 h, 37 $^{\circ}\text{C}$), LDL oxidized with SIN-1 (2 mM) + 200 units/ml superoxide dismutase (SOD, 5 h, 37 $^{\circ}\text{C}$), or LDL oxidized with metmyoglobin (30 μM , 16 h, 37 $^{\circ}\text{C}$), 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.

Condition	Fold change in [total glutathione]	[Lipid peroxide] (nmol/mg)
Control	1	NA
Copper-oxidized LDL (150 $\mu\text{g}/\text{ml}$)	$3.8 \pm 0.75^*$	118.2 ± 2.2
nLDL (150 $\mu\text{g}/\text{ml}$)	0.8 ± 0.25	17.9 ± 1.9
SIN-1-oxidized LDL (150 $\mu\text{g}/\text{ml}$)	$3.0 \pm 1.0^*$	73.7 ± 11.8
SIN-1 + SOD-oxidized LDL (150 $\mu\text{g}/\text{ml}$)	1.4 ± 0.75	13.5 ± 0.22
Metmyoglobin-oxidized LDL (150 $\mu\text{g}/\text{ml}$)	$4.6 \pm 1.5^*$	129.2 ± 1.7
30 μM Metmyoglobin	0.6 ± 0.25	NA
LysoPC		
25 μM	0.9 ± 0.75	
50 μM	1.0 ± 0.75	
100 μM	0.9 ± 0.5	
13-HPODE		
20 μM	$1.4 \pm 0.1^*$	
40 μM	$1.63 \pm 0.07^*$	
HNE		
10 μM	1.35 ± 0.5	
30 μM	$3.30 \pm 1.25^*$	

present in oxLDL (Table 1). Lipopolysaccharide (LPS) is also known to have effects on GSH synthesis [36]. LPS concentrations were determined [using the *Limulus* (horseshoe crab) amoebocyte lysate assay] in the same LDL solutions that were exposed to BAEC (i.e. sterile-filtered and at a concentration of 150 μg of protein/ml). LPS concentrations in both native and oxLDL were at the limit of detection, being 0.09 ± 0.02 EU/ml (mean $\pm$ S.E.M., $n = 3$) for native LDL and 0.09 ± 0.04 EU/ml for ox-LDL (1 EU = 200 pg of endotoxin). Since no significant difference was observed in LPS levels between the native and oxLDL, it was concluded that these low levels in the oxLDL preparations were not contributing to the induction of GSH synthesis.

The increase in cellular GSH and the induction of GCLC by oxLDL in macrophages has been attributed to the lipid hydroperoxides [4]. To address the role of hydroperoxides in the increase in total glutathione in BAEC, we exposed cells to (13S)-hydroperoxyoctadecadienoic acid (13-HPODE), which is produced by lipoxygenase-catalysed oxidation of linoleic acid. Moreover, the amount of hydroperoxides formed in LDL oxidation by Cu^{2+} or metmyoglobin was assessed (Table 1). Exposure of the cells to 20 μM 13-HPODE resulted in a small, but significant (1.4-fold), increase in total glutathione. This concentration corresponds approximately to the amount of hydroperoxides found in 150 $\mu\text{g}/\text{ml}$ LDL oxidized by metmyoglobin or Cu^{2+} , which results in an about 4-fold induction of total glutathione. These data suggest that lipid hydroperoxides, although capable of inducing GSH synthesis, have a minor impact on the increase in cellular glutathione. We also tested the effects of HNE, a known inducer of GSH in lung epithelial cells [6], on endothelial cell GSH levels. HNE induced GSH significantly at a concentration of 30 μM (Table 1), which is 20–100 times higher than the concentration that oxLDL is reported to contain [37].

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 $\pm$ 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

Figure 4 Inhibition of GSH synthesis causes oxLDL cytotoxicity

Cells were treated with BSO (250 μM) for 24 h prior to and during exposure to oxLDL (750 μg /ml). (A) 24 h viability was determined as the loss of LDH to the medium and (B) the total glutathione was measured in the cell lysate. Control cells were treated with Cu^{2+} alone. Results are means $\pm$ S.E.M. for triplicate wells, each containing 10^5 cells (* P < 0.01 relative to control).

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 suggests that oxidized arachidonyl phospholipids 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

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

(A) Cells were treated with oxLDL (100 μ g/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 $\pm$ S.E.M. for triplicate wells, each containing 10^5 cells (* P < 0.01 relative to control). (B and C) Cells were treated with oxLDL (150 μ g/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.

treated with BSO, supporting an essential role of GSH in cytoprotection against oxLDL.

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 μ M 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 μ g/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 μ M) for 24 h. (B) Cells were incubated with or without ox- or n-PAPC (100 μ g/ml) for 16 h before the medium was changed and the cells were exposed to DMNQ (20 μ M) 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 $\pm$ 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 μ M. In these experiments a concentration of 20 μ M 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 polar-lipid 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 leucocyte 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 (≈ 150 ng/ml) 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 cytoprotective 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 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 μ M 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 μ M) 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 R01ES/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

- Smith, C. V., Jones, D. P., Guenther, T. M., Lash, L. H. and Lauterburg, B. H. (1996) Compartmentation of glutathione: implications for the study of toxicity and disease. *Toxicol. Appl. Pharmacol.* **140**, 1–12
- Wild, A. C. and Mulcahy, R. T. (2000) Regulation of γ -glutamylcysteine synthetase subunit gene expression: insights into transcriptional control of antioxidant defenses. *Free Radical Res.* **32**, 281–301
- Darley-Usmar, V. M., Severn, A., O'Leary, V. J. and Rogers, M. (1991) Treatment of macrophages with oxidized low-density lipoprotein increases their intracellular glutathione content. *Biochem. J.* **278**, 429–434
- Shen, L. and Sevanian, A. (2001) OxLDL induces macrophage γ -GCS-HS protein expression: a role for oxLDL-associated lipid hydroperoxide in GSH synthesis. *J. Lipid Res.* **42**, 813–823
- Gotoh, N., Graham, A., Nikl, E. and Darley-Usmar, V. M. (1993) Inhibition of glutathione synthesis increases the toxicity of oxidized low-density lipoprotein to human monocytes and macrophages. *Biochem. J.* **296**, 151–154
- Liu, R. M., Gao, L., Choi, J. and Forman, H. J. (1998) γ -glutamylcysteine synthetase: mRNA stabilization and independent subunit transcription by 4-hydroxy-2-nonenal. *Am. J. Physiol.* **275**, L861–L869
- Rosenblat, M. and Aviram, M. (1998) Macrophage glutathione content and glutathione peroxidase activity are inversely related to cell-mediated oxidation of LDL: *in vitro* and *in vivo* studies. *Free Radicals Biol. Med.* **24**, 305–317
- Cathcart, M. K. and Folcik, V. A. (2000) Lipoxigenases and atherosclerosis: protection versus pathogenesis. *Free Radicals Biol. Med.* **28**, 1726–1734
- Ma, X. L., Lopez, B. L., Liu, G. L., Christopher, T. A., Gao, F., Guo, Y., Feuerstein, G. Z., Ruffolo, R. R. J., Barone, F. C. and Yue, T. L. (1997) Hypercholesterolemia impairs a detoxification mechanism against peroxynitrite and renders the vascular tissue more susceptible to oxidative injury. *Circ. Res.* **80**, 894–901
- Cuzzocrea, S., Zingarelli, B., O'Connor, M., Salzman, A. L. and Szabo, C. (1998) Effect of L-buthionine-(S,R)-sulphoximine, an inhibitor of γ -glutamylcysteine synthetase on peroxynitrite- and endotoxic shock-induced vascular failure. *Br. J. Pharmacol.* **123**, 525–537
- Graham, A., Hogg, N., Kalyanaraman, B., O'Leary, V., Darley-Usmar, V. and Moncada, S. (1993) Peroxynitrite modification of low-density lipoprotein leads to recognition by the macrophage scavenger receptor. *FEBS Lett.* **330**, 181–185
- Hegyi, L., Skepper, J. N., Cary, N. R. and Mitchinson, M. J. (1996) Foam cell apoptosis and the development of the lipid core of human atherosclerosis. *J. Pathol.* **180**, 423–429
- Lizard, G., Gueldry, S., Sordet, O., Monier, S., Athias, A., Miquet, C., Bessede, G., Lemaire, S., Solary, E. and Gambert, P. (1998) Glutathione is implied in the control of 7-ketocholesterol-induced apoptosis, which is associated with radical oxygen species production. *FASEB J.* **12**, 1651–1663
- Parthasarathy, S., Santanam, N., Ramachandran, S. and Meilhack, O. (2000) Potential role of oxidized lipids and lipoproteins in antioxidant defense. *Free Radical Res.* **33**, 197–215
- Luoma, J. S., Stralin, P., Marklund, S. L., Hiltunen, T. P., Sarkioja, T. and Yla-Herttuala, S. (1998) Expression of extracellular SOD and iNOS in macrophages and smooth muscle cells in human and rabbit atherosclerotic lesions: colocalization with epitopes characteristic of oxidized LDL and peroxynitrite-modified proteins. *Arterioscler. Thromb. Vasc. Biol.* **18**, 157–167
- Radi, R., Beckman, J. S., Bush, K. M. and Freeman, B. A. (1991) Peroxynitrite-induced membrane lipid peroxidation: the cytotoxic potential of superoxide and nitric oxide. *Arch. Biochem. Biophys.* **288**, 481–487
- Podrez, E. A., Abu-Soud, H. M. and Hazen, S. L. (2000) Myeloperoxidase-generated oxidants and atherosclerosis. *Free Radicals Biol. Med.* **28**, 1717–1725
- Savenkova, M. L., Mueller, D. M. and Heinecke, J. W. (1994) Tyrosyl radical generated by myeloperoxidase is a physiological catalyst for the initiation of lipid peroxidation in low density lipoprotein. *J. Biol. Chem.* **269**, 20394–20400
- Podrez, E. A., Schmitt, D., Hoff, H. F. and Hazen, S. L. (1999) Myeloperoxidase-generated reactive nitrogen species convert LDL into an atherogenic form *in vitro*. *J. Clin. Invest.* **103**, 1547–1560
- Schmitt, D., Shen, Z., Zhang, R., Colles, S. M., Wu, W., Salomon, R. G., Chen, Y., Chisolm, G. M. and Hazen, S. L. (1999) Leucocytes utilize myeloperoxidase-generated nitrating intermediates as physiological catalysts for the generation of biologically active oxidized lipids and sterols in serum. *Biochemistry* **38**, 16904–16915
- Podrez, E. A., Febbraio, M., Sheibani, N., Schmitt, D., Silverstein, R. L., Hajjar, D. P., Cohen, P. A., Frazier, W. A., Hoff, H. F. and Hazen, S. L. (2000) Macrophage scavenger receptor CD36 is the major receptor for LDL modified by monocyte-generated reactive nitrogen species. *J. Clin. Invest.* **105**, 1095–1108
- Watson, A. D., Subbanagounder, G., Welsbie, D. S., Faull, K. F., Navab, M., Jung, M. E., Fogelman, A. M. and Berliner, J. A. (1999) Structural identification of a novel pro-inflammatory epoxyisoprostane phospholipid in mildly oxidized low density lipoprotein. *J. Biol. Chem.* **274**, 24787–24798
- Zhang, R., Brennan, M. L., Fu, X., Aviles, R. J., Pearce, G., Penn, M. S., Topol, E. J., Sprecher, D. S. and Hazen, S. L. (2001) Myeloperoxidase as an independent marker of coronary artery disease. *JAMA* **286**, 2136–2142
- Leonarduzzi, G., Arkan, M. C., Basaga, H., Chiarotto, E., Sevanian, A. and Poli, G. (2000) Lipid oxidation products in cell signalling. *Free Radicals Biol. Med.* **28**, 1370–1378
- Cho, S., Hazama, M., Urata, Y., Goto, S., Horiuchi, S., Sumikawa, K. and Kondo, T. (1999) Protective role of glutathione synthesis in response to oxidized low density lipoprotein in human vascular endothelial cells. *Free Radicals Biol. Med.* **26**, 589–602
- White, C. R., Darley-Usmar, V., Berrington, W. R., McAdams, M., Gore, J. Z., Thompson, J. A., Parks, D. A., Tarpey, M. M. and Freeman, B. A. (1996) Circulating plasma xanthine oxidase contributes to vascular dysfunction in hypercholesterolemic rabbits. *Proc. Natl. Acad. Sci. U.S.A.* **93**, 8745–8749
- Chung, B. H., Wilkinson, T., Geer, J. C. and Segrest, J. P. (1980) Preparative and quantitative isolation of plasma lipoproteins: rapid, single discontinuous density gradient ultracentrifugation in a vertical rotor. *J. Lipid Res.* **21**, 284–291
- Darley-Usmar, V. M., Hogg, N., O'Leary, V. J., Wilson, M. T. and Moncada, S. (1992) The simultaneous generation of superoxide and nitric oxide can initiate lipid peroxidation in human low density lipoprotein. *Free Radicals Res. Commun.* **17**, 9–20
- Bligh, E. G. and Dyer, W. J. (1959) A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.* **37**, 911–917
- Jiang, Z. Y., Hunt, J. V. and Wolff, S. P. (1992) Ferrous ion oxidation in the presence of xylenol orange for detection of lipid hydroperoxide in low density lipoprotein. *Anal. Biochem.* **202**, 384–389
- Schutte, B., Nuydens, R., Geerts, H. and Ramaekers, F. (1998) Annexin V binding assay as a tool to measure apoptosis in differentiated neuronal cells. *J. Neurosci. Methods* **86**, 63–69
- Tietze, F. (1969) Enzymic method for quantitative determination of nanogram amounts of total and oxidized glutathione: applications to mammalian blood and other tissues. *Anal. Biochem.* **27**, 502–522
- Moellering, D., McAndrew, J., Patel, R. P., Cornwell, T., Lincoln, T., Cao, X., Messina, J. L., Forman, H. J., Jo, H. and Darley-Usmar, V. M. (1998) Nitric oxide-dependent induction of glutathione synthesis through increased expression of γ -glutamylcysteine synthetase. *Arch. Biochem. Biophys.* **358**, 74–82
- Tarpey, M. M., White, C. R., Suarez, E., Richardson, G., Radi, R. and Freeman, B. A. (1999) Chemiluminescent detection of oxidants in vascular tissue. Lucigenin but not coelenterazine enhances superoxide formation. *Circ. Res.* **84**, 1203–1211
- Sata, M. and Walsh, K. (1998) Oxidized LDL activates *f*_{as}-mediated endothelial cell apoptosis. *J. Clin. Invest.* **102**, 1682–1689
- Vieira, O., Escargueil-Blanc, I., Meilhac, O., Basile, J. P., Laranjinha, J., Almeida, L., Salvayre, R. and Negre-Salvayre, A. (1998) Effect of dietary phenolic compounds on apoptosis of human cultured endothelial cells induced by oxidized LDL. *Br. J. Pharmacol.* **123**, 565–573
- Spolarics, Z. and Wu, J. X. (1997) Role of glutathione and catalase in H₂O₂ detoxification in LPS-activated hepatic endothelial and Kupffer cells. *Am. J. Physiol.* **273**, G1304–G1311
- Esterbauer, H., Gebicki, J., Puhl, H. and Jurgens, G. (1992) The role of lipid peroxidation and antioxidants in oxidative modification of LDL. *Free Radicals Biol. Med.* **13**, 341–390
- Therond, P., Abella, A., Laurent, D., Couturier, M., Chalas, J., Legrand, A. and Lindenbaum, A. (2000) *In vitro* study of the cytotoxicity of isolated oxidized lipid low-density lipoproteins fractions in human endothelial cells: relationship with the glutathione status and cell morphology. *Free Radicals Biol. Med.* **28**, 585–596
- Hogg, N., Rice-Evans, C., Darley-Usmar, V., Wilson, M. T., Paganga, G. and Bourne, L. (1994) The role of lipid hydroperoxides in the myoglobin-dependent oxidation of LDL. *Arch. Biochem. Biophys.* **314**, 39–44

- 40 Moinova, H. R. and Mulcahy, R. T. (1998) An electrophile responsive element (EpRE) regulates β -naphthoflavone induction of the human γ -glutamylcysteine synthetase regulatory subunit gene. Constitutive expression is mediated by an adjacent AP-1 site. *J. Biol. Chem.* **273**, 14683–14689
- 41 Mulcahy, R. T., Wartman, M. A., Bailey, H. H. and Gipp, J. J. (1997) Constitutive and β -naphthoflavone-induced expression of the human γ -glutamylcysteine synthetase heavy subunit gene is regulated by a distal antioxidant response element/TRE sequence. *J. Biol. Chem.* **272**, 7445–7454
- 42 Shi, M. M., Kugelman, A., Iwamoto, T., Tian, L. and Forman, H. J. (1994) Quinone-induced oxidative stress elevates glutathione and induces γ -glutamylcysteine synthetase activity in rat lung epithelial L2 cells. *J. Biol. Chem.* **269**, 26512–26517
- 43 Siow, R. C., Sato, H., Leake, D. S., Ishii, T., Bannai, S. and Mann, G. E. (1999) Induction of antioxidant stress proteins in vascular endothelial and smooth muscle cells: protective action of vitamin C against atherogenic lipoproteins. *Free Radical Res.* **31**, 309–318
- 44 Choi, J., Liu, R. M., Kundu, R. K., Sangiorgi, F., Wu, W., Maxson, R. and Forman, H. J. (2000) Molecular mechanism of decreased glutathione content in human immunodeficiency virus type 1 Tat-transgenic mice. *J. Biol. Chem.* **275**, 3693–3698
- 45 Wasserman, W. W. and Fahl, W. E. (1997) Functional antioxidant responsive elements. *Proc. Natl. Acad. Sci. U.S.A.* **94**, 5361–5366
- 46 Ishikawa, K., Navab, M., Leitinger, N., Fogelman, A. M. and Lusis, A. J. (1997) Induction of heme oxygenase-1 inhibits the monocyte transmigration induced by mildly oxidized LDL. *J. Clin. Invest.* **100**, 1209–1216
- 46a Levonen, A.-L., Dickinson, D. A., Moellering, D. R., Mulcahy, R. T., Forman, H. J. and Darley-Usmar, V. M. (2001) Biphasic effects of 15-deoxy- $\Delta^{12,14}$ -prostaglandin J_2 on glutathione induction and apoptosis in human endothelial cells. *Arterioscler. Thromb. Vasc. Biol.* **21**, 1846–1851
- 47 Li, N., Venkatesan, M. I., Miguel, A., Kaplan, R., Gujuluva, C., Alam, J. and Nel, A. (2000) Induction of heme oxygenase-1 expression in macrophages by diesel exhaust particle chemicals and quinones via the antioxidant-responsive element. *J. Immunol.* **165**, 3393–3401
- 48 Misra, P., Srivastava, S. K., Singhal, S. S., Awasthi, S., Awasthi, Y. C. and Boor, P. J. (1995) Glutathione S-transferase 8–8 is localized in smooth muscle cells of rat aorta and is induced in an experimental model of atherosclerosis. *Toxicol. Appl. Pharmacol.* **133**, 27–33
- 49 Griendling, K. K., Sorescu, D. and Ushio-Fukai, M. (2000) NAD(P)H oxidase: role in cardiovascular biology and disease. *Circ. Res.* **86**, 494–501
- 50 Colles, S. M., Irwin, K. C. and Chisolm, G. M. (1996) Roles of multiple oxidized LDL lipids in cellular injury: dominance of 7 β -hydroperoxycholesterol. *J. Lipid Res.* **37**, 2018–2028

Received 19 September 2001; accepted 28 November 2001