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Original Contribution

Molecular mechanism of glutathione-mediated protection from oxidized low-density lipoprotein-induced cell injury in human macrophages: Role of glutathione reductase and glutaredoxin

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Abstract

Macrophage death is a hallmark of advanced atherosclerotic plaque, and oxidized low-density lipoprotein (OxLDL) found in these lesions is believed to contribute to macrophage injury. However, the underlying mechanisms of this phenomenon are only poorly understood. Here we show that in human monocyte-derived macrophages, OxLDL depleted intracellular glutathione (GSH) and inhibited glutathione reductase, resulting in a marked diminution of the glutathione/glutathione disulfide ratio. In the absence of OxLDL, an 80% depletion of intracellular GSH levels did not affect cell viability, but glutathione depletion dramatically increased OxLDL-induced cell death. Conversely, supplementation of intracellular GSH stores with glutathione diethyl ester substantially diminished OxLDL toxicity. OxLDL also promoted protein-*S*-glutathionylation, which was increased in macrophages pretreated with the glutathione reductase inhibitor BCNU. Knockdown experiments with siRNA directed against glutathione reductase and glutaredoxin showed that both enzymes are essential for the protection of macrophages against OxLDL. Finally, the peroxyl-radical scavenger Trolox did not prevent GSH depletion but completely blocked OxLDL-induced protein-*S*-glutathionylation and cell death. These data suggest that OxLDL promotes ROS formation and protein-*S*-glutathionylation by a mechanism independent from its effect on GSH depletion. Neither mechanism was sufficient to induce macrophage injury, but when stimulated concurrently, these pathways promoted the accumulation of protein-glutathione mixed disulfides and cell death.

Keywords: Macrophage; Atherosclerosis; Cell death; Glutathione; Glutaredoxin; Glutathione reductase

Introduction

The appearance of lipid-rich necrotic cores is a manifestation of advanced atherosclerotic plaques and a compelling argument that cell injury contributes to the progression and possibly even the development of early atherosclerotic lesions. While endothelial cell injury and death have long been recognized as initial events of atherogenesis [1,2], a large body of evidence now suggests that macrophage injury and macrophage foam cell death contribute to lesion progression and the formation of the necrotic core [3–5]. The cells found in the proximity of necrotic cores have been identified primarily as macrophage-derived foam cells, many of which show signs of cell injury and death [6,7]. The necrotic cores themselves contain macrophage debris,

Abbreviations: BCNU, 1,3-bis[2-chloroethyl]-1-nitrosourea; BSO, L-buthionine [*S,R*]-sulfoximine; DCFH, 6-carboxy-2′,7′-dichlorodihydrofluorescein diacetate di(acetoxymethyl) ester; DEM, diethyl maleate; diamide, 1,1′-azobis (*N*,*N*-dimethylformamide); DPPD, *N*,*N*′-diphenyl-1,4-phenylene diamine; DTT, dithiothreitol; FITC, fluorescein thioisocyanate; GR, glutathione reductase; Grx, glutaredoxin; GSH, reduced glutathione; GSSG, glutathione disulfide (oxidized glutathione); HPLC, high-performance liquid chromatography; 4-HNE, 4-hydroxynonenal; KP_i, potassium phosphate buffer; LIP, labile iron pools; NaP_i, sodium phosphate buffer; OxLDL, oxidized low-density lipoprotein; PBS, phosphate-buffered saline; PSSG, protein-conjugated glutathione, i.e., mixed disulfides of glutathione and protein-cysteine residues; ROS, reactive oxygen species; TBARS, thiobarbituric acid-reactive substances.

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but not smooth muscle actin [8], suggesting that macrophages and macrophage-derived foam cells are the primary source of the material found in these acellular areas. The mechanisms underlying macrophage death in vivo are still unclear.

Atherosclerotic plaque contains both apoptotic and necrotic cells, but the majority of cells in human atherosclerotic plaque appear to undergo cell death by oncosis [9]. Taken together, the data suggest that in atherosclerotic lesions macrophages and in particular macrophage-derived foam cells appear to undergo cell lysis. Regardless of whether macrophage lysis occurs postapoptosis due to insufficient clearance of apoptotic cells and subsequent secondary necrosis, or as the result of nonapoptotic cell death, loss of plasma membrane integrity and the concomitant release of the noxious cell contents are likely to play an important role in promoting vascular inflammation, necrotic core formation, and plaque destabilization. In this context it is important to note that plaque ruptures associated with myocardial infarction are more likely to occur in lesions with large necrotic cores and a high concentration of macrophage-derived foam cells [10].

Oxidized low-density lipoprotein (OxLDL) is a prominent component of atherosclerotic lesions [11] and it is believed to be a major factor in the development and progression of atherosclerosis [12]. OxLDL is cytotoxic for all cells involved in atherogenesis, including macrophages and macrophage-derived foam cells, and a large body of evidence suggests that OxLDL may contribute to cell death in atherosclerotic lesions [4,5,13]. In macrophage-like cell lines, OxLDL stimulates GSH synthesis [14–17] and pharmacological depletion of cellular GSH enhanced OxLDL cytotoxicity [16], suggesting that GSH may protect macrophages from OxLDL-induced cell injury. We recently reported that OxLDL induces cell death in human monocyte-derived macrophages in a caspase-independent manner and that both metal-dependent and metal-independent oxidation renders LDL cytotoxic for human macrophages [18]. We also showed that OxLDL-induced macrophage death is mediated by peroxides and/or peroxyl radicals. However, peroxide formation alone does not appear to be sufficient to promote macrophage death, suggesting that a second contributory mechanism is required. Here, we present evidence that this second mechanism involves the disruption of the intracellular glutathione redox buffer initiated by the OxLDL-induced depletion of reduced glutathione (GSH) and inhibition of glutathione reductase (GR).

Methods

Chemicals

1,3-Bis[2-chloroethyl]-1-nitrosourea (BCNU), Triton X-100, dithiothreitol (DTT), *N*-ethylmaleimide, *o*-phthalaldehyde, *N*, *N*′-diphenyl-1,4-phenylene diamine (DPPD), L-buthionine- [*S*, *R*]-sulfoximine (BSO, diethyl maleate, glutathione (GSH), and glutathione disulfide (GSSG) were purchased from Sigma-Aldrich (St. Louis, MO). Acetonitrile, methanol, and propionic acid were purchased from Fischer Scientific (Pittsburgh, PA). 6-Carboxy-2′,7′-dichlorodihydrofluorescein diacetate di(acetoxymethyl) ester (DCFH) was from Molecular Probes/Invitrogen (Carlsbad, CA).

Isolation and culture of human monocyte-derived macrophages

Human mononuclear cells were isolated from buffy coats by density gradient centrifugation, purified, and cultured for 2 weeks in Teflon bags, as described elsewhere [19]. Whole blood samples were obtained through the Central Kentucky Blood Center in Lexington and collected from apparently healthy male and female volunteers. Mature macrophages were plated on Aclar (22-mm-diameter, transparent fluorinated-chlorinated thermoplastic film, ProPlastics) in 12-well plates at a density of 0.15×10^6 cells/well for all other experiments. The cell culture medium, referred to subsequently as "culture medium," consisted of RPMI (Gibco BRL) supplemented with 2 mM L-alanyl-L-glutamine (GLUTAMAX-1, Gibco BRL), 1% v/v nonessential amino acids (Gibco BRL), 1 mM sodium pyruvate (Gibco BRL), penicillin G/streptomycin (0.1 U/ml and 0.1 mg/ml, respectively, Gibco BRL,), and 10 mM Hepes (Fluka). All solutions were routinely tested for endotoxin. Endotoxin levels of the culture medium and all buffers were below 0.03 EU/ml. Cells were plated in culture medium supplemented with 5% human AB serum (Nabi). After 2 h, nonadherent cells were removed through washing with culture medium. Macrophages were incubated in culture medium containing 5% human AB serum for 48 h prior to the experiments.

Lipoproteins

Plasma obtained from six male donors was pooled, aliquoted, and kept at −20°C. Human LDL was prepared freshly for each experiment and was isolated under lipopolysaccharide-free conditions by discontinuous gradient density and flotation ultracentrifugation in a TL-100 ultracentrifuge (Beckman Instruments) equipped with a TLA-100.4 fixed-angle rotor, as described previously [20]. LDL was concentrated by ultrafiltration in Centricon-100 concentrators (Amicon) and was further purified by gel-filtration chromatography on excellulose GF-5 columns (Pierce). LDL was diluted in phosphate-buffered saline (PBS) at a concentration of 3 mg/ml and was oxidized for 24 h at 37°C with $CuSO_4$, as described by Esterbauer et al. [21]. OxLDL, prepared freshly for each experiment, was concentrated by ultrafiltration in Centricon-100 concentrators (Amicon), purified by gel filtration chromatography on excellulose GF-5 columns (Pierce). OxLDL was analyzed for thiobarbituric acid-reactive substances (TBARS), lipid peroxides, and electrophoretic mobility, as published previously [22]. The range for TBARS, lipid peroxides, and relative electrophoretic mobility was 8.0–8.4 nmol/mg protein, 140–152 nmol/mg protein, and 2.8–3.0, respectively. Protein concentrations of all LDL solutions were determined with bicinchoninic acid (Pierce) using bovine serum albumin as a standard.

Cytotoxicity assay

Membrane integrity was assessed with the [^{3}H]adenine release method developed by Reid and Mitchinson [23], as

described previously [18]. Briefly, macrophages were loaded with [8-^{3}H]adenine (1 μCi/ml, Amersham, Switzerland) in culture medium with 5% human autologous serum for 2 h and washed twice with PBS for 5 min at 37°C. Cells were then incubated for 24 h, unless stated otherwise with culture medium either alone or supplemented with OxLDL. Loss of membrane integrity was measured as the percentage of radioactivity released into the supernatant. Intracellular radioactivity was measured after cell lysis with 1% (by vol) Triton X-100. Radioactivity was determined by scintillation counting. Total radioactivity per well was always proportional to cell number, as determined by DNA measurement. Control experiments also confirmed that adenine loading did not interfere with any of our other assays. For selected experiments, macrophages were stained for 45 min in the dark with 2 μM ethidium homodimer-1 (Molecular Probes, Eugene, OR) to verify membrane integrity as described previously [18].

Determination of cellular glutathione

Macrophages were washed with PBS and harvested by scraping. Samples were prepared and analyzed as described previously [24]. Briefly, each sample was split into three 500-μl aliquots, one for the determination of DNA as a measure of cell number, and one each for the determination of glutathione disulfide and total glutathione (GSH + GSSG). The samples designated for GSSG determination were supplemented with *N*-ethylmaleimide (7.4 mM) to alkylate free thiol groups. Proteins were precipitated with 100 μl cold 18% perchloric acid and supernatants were neutralized with 1 M KP_i (pH 7.0). Samples were diluted with 0.1 M KP_i (pH 7.0) and reduced with 10 mM dithiothreitol for 60 min at room temperature. Glutathione was derivatized in the presence of 11 mM *o*-phthalaldehyde and separated by reverse-phase HPLC. HPLC analysis was performed on a Jasco HPLC system equipped with a spectrofluorometer (FP-920, Jasco Inc.) set to an excitation wavelength of 340 nm and an emission wavelength of 420 nm. Glutathione was separated isocratically on a Brownlee 3-cm C18 ODS guard column (5 μm) and a Brownlee 22-cm C18 ODS analytical column (5 μm) with 21 mM propionate buffer (in 35 mM NaP_i, pH 6.5)/acetonitrile (95/5 by vol) at a flow rate of 1.2 ml/min. Levels of reduced glutathione were calculated as the difference between GSH + GSSG and GSSG.

Determination of protein-S-glutathionylation

Macrophages were washed with PBS and harvested by scraping; each sample was split into 2 aliquots, one for the determination of DNA (500 μl) and one for the determination of protein-bound glutathione (1000 μl, PSSG) as described previously [24]. Proteins were precipitated as described above. Protein pellets were resuspended in 0.5 M NaOH and neutralized with 1 M KP_i (pH 7.0). Glutathione was released by reduction with 7 mM dithiothreitol for 1 h at room temperature. Proteins were precipitated, and the supernatants were analyzed for glutathione, as described above.

Glutathione reductase activity assay

Macrophage lysates were prepared with 50 mM KP_i, pH 7.5, containing 1 mM EDTA and 1% Triton X-100 [24]. The assay was performed at 37°C in 50 mM KP_i, pH 7.5, containing 1 mM EDTA, BSA (1 mg/ml), and 333 μM NADPH. The enzymatic reaction was started by the addition of 1 mM GSSG and absorbance was monitored at 340 nm for 15 min on a VERSAmax plate reader (Molecular Devices, Sunnyvale, CA). Glutathione reductase (GR) from Baker's yeast served as a standard.

Glutaredoxin activity assay

Macrophages were lysed in 50 μM Tris-HCl, 2.2 μM sodium phosphate, 0.7 μM potassium phosphate, 369 μM NaCl, 1.35 μM KCl, 1% (w/v) Triton X-100, 10 μg/ml leupeptin, 20 μg/ml aprotinin, and 2 μl/ml DMSO, and glutaredoxin (Grx) activity was assayed using the mixed disulfide [^{35}S]glutathionyl-*S*-*S*-$(CH_2)_2$-CO-NH_2-derivative of *S*-carboxymethyl bovine serum albumin (BSA-SSG[^{35}S]) as previously described [25].

Measurement of cellular DNA

The quantity of DNA was determined fluorometrically using the PicoGreen DNA Quantitation Kit (Molecular Probes). Fluorescence was measured in a FUSION plate reader (Packard) set to an excitation wavelength of 485 nm and an emission wavelength of 535 nm.

Generation of small interfering RNA (siRNA)

siRNA directed against glutathione reductase and glutaredoxin, respectively, was prepared with the Dicer siRNA Generation Kit (Gene Therapy Systems, Eugene, OR). Template DNA for transcription was generated by PCR from plasmids carrying either human glutathione reductase obtained from Dr. Dieter Werner (University of Heidelberg, Germany), or human glutaredoxin as described previously [25]. Double-stranded RNA was synthesized with the T_7 enzyme mix and 22 bp siRNA was prepared using recombinant Dicer enzyme according to the manufacturer's instructions. The siRNA were column-purified and RNA concentrations were measured with the RiboGreen RNA Quantification Kit (Molecular Probes). Human macrophages were transfected for 6 h at 37°C with 0.5 μg siRNA. Transfection was performed under serum-free conditions with the GeneSilencer siRNA transfection reagent (Gene Therapy Systems, Eugene, OR). FITC-labeled siRNA directed against luciferase GL2 (Dharmacon, Lafayette, CO) served as both a transfection and a negative control in siRNA experiments.

Statistics

All experiments were performed in triplicates and repeated at least four times unless stated otherwise. All data are presented as mean ± SE. Data were statistically analyzed using ANOVA. Unless indicated otherwise, multiple group comparisons versus

control group were performed using the Holm-Sidak test. Results were considered significant at the $p < 0.05$ level.

Results

OxLDL alters the glutathione thiol redox state by depleting reduced glutathione

The GSH/GSSG ratio is one of the principal determinants of the cellular redox environment, and alterations in the redox environment can lead to cellular dysfunction and cell death [26]. To determine if OxLDL-induced cell injury involves changes in the glutathione redox state, we measured cell viability and GSH and GSSG levels in human macrophages treated for 24 h with OxLDL at concentrations ranging from 10 to 75 μg/ml. OxLDL induced a dose-dependent decrease in the GSH/GSSG ratio (Fig. 1, open symbols), indicating a progressive shift to a more oxidizing intracellular environment. Even at concentrations as low as 10 μg/ml, we routinely observed a decrease in the GSH/GSSG ratio; however, the difference did not reach statistical significance. At 50 μg/ml OxLDL, the GSH/GSSG ratio was decreased by 47%, from 40.4 to 21.4, compared to control cells, yet cell viability was not significantly decreased (Fig. 1, closed symbols). In the presence of 75 μg/ml OxLDL, we measured a GSH/GSSG ratio of 12.6 (69% decrease), but at this stage macrophage viability was significantly decreased; from 94 to 52%. Therefore the value of the GSH/GSSG ratio is only an estimate for the surviving cells because it assumes that all GSSG and GSH had leaked from the dying cells. We showed previously that the dose-response curve for OxLDL-induced macrophage injury shifts significantly to the left when the incubation time is increased from 24 to 48 and 72 h [18], indicating that the gradual collapse of the glutathione redox state induced by OxLDL precedes and may directly contribute to macrophage death.

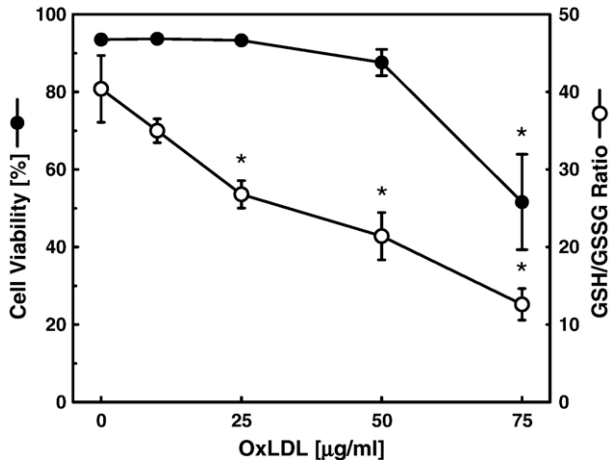

Fig. 1. OxLDL-induced decrease in GSH/GSSG ratio precedes loss of macrophage viability. Monocytes were isolated from four individuals and cultured for 2 weeks in human AB serum as described under Methods. Mature macrophages were then loaded with [^{3}H]adenine for 2 h, washed, and incubated with OxLDL at the indicated concentrations. After 24 h, cells were harvested and the cellular total non-protein-bound glutathione (GSH_{tot}) and glutathione disulfide (GSSG) contents were measured. For the determination of the GSH/GSSG ratio (open circles), cellular GSH levels were calculated as the difference between (GSH_{tot}-2 [GSSG]). Macrophage viability (closed circles) was determined as a percentage of radiolabel released from cells as described under Methods. Results are expressed as mean ± SE of four independent experiments performed in triplicates. * $p < 0.05$ vs no OxLDL added.

Analysis of the intracellular GSH and GSSG levels revealed that the OxLDL-induced decrease of the GSH/GSSG ratio appears to be primarily due to the depletion of GSH (Fig. 2, closed symbols) rather than increased GSSG accumulation (Fig. 2, open symbols). With increasing OxLDL concentrations, we also observed a trend toward lower GSSG levels, but the GSSG curve, at least at low OxLDL concentrations, did not parallel the loss in GSH. The decrease in GSSG was statistically significant at 75 μg/ml OxLDL, but partial cell leakage due to cell death observed under these conditions may have contributed to the decrease in GSSG (see Fig. 1).

To determine whether GSH efflux could account for the loss of cellular GSH, we also measured GSH_{tot} in the cell supernatant of macrophages treated with OxLDL. In the absence of OxLDL, cell supernatants routinely had higher GSH_{tot} levels than the corresponding culture medium incubated for 24 h without cells (1.97 ± 0.06 μM, dotted line), indicating that human macrophages release glutathione. Preventing the degradation of extracellular GSH by blocking γ-glutamyltranspeptidase with acivicin (75 μM) increased extracellular GSH_{tot} levels 3-fold to 4-fold (not shown). This result confirms that macrophages secrete significant amounts of intracellular glutathione. However, OxLDL did not significantly increase extracellular GSH_{tot}. In fact, OxLDL at 50 and 75 μg/ml decreased extracellular GSH_{tot} (Fig. 3), suggesting that the loss in intracellular GSH induced by OxLDL in macrophages (Fig. 3, insert) cannot be explained by increased GSH efflux.

Next, we examined whether alkylation of GSH could explain the depletion of GSH induced by OxLDL. To this end, we compared the effect of OxLDL on the macrophage GSH redox state with that observed in macrophages treated for 24 h either with the thiol oxidant diamide (0.5 mM) or with 4-hydroxynonenal (4-HNE, 50 μM), which in a reaction catalyzed by GSH-*S*-transferases forms GSH conjugates, resulting in the alkylation of GSH [27]. At the chosen concentrations, both 4-HNE and diamide induced macrophage death to a similar extent as 75 μg/ml OxLDL, i.e., 45–65%, and all three compounds decrease the GSH/GSSG ratio (Table 1). Both OxLDL and 4-HNE decrease GSH_{tot} by 86–91%. In contrast to OxLDL and 4-HNE, diamide treatment did not decrease GSH_{tot}levels. Instead, the decrease in the GSH/GSSG ratio observed in diamide-treated macrophages was caused by a 3.2-fold increase in GSSG. These data suggest that conjugation of GSH rather than GSH oxidation mediates the OxLDL-induced collapse of the GSH/GSSG ratio in human macrophages.

Glutathione depletion is not sufficient to promote macrophage death

To determine if GSH depletion is sufficient to promote macrophage death, human macrophages were pretreated for 2 h with the thiol alkylating agent diethyl maleate (DEM). Furthermore, to prevent GSH synthesis and the replenishing of

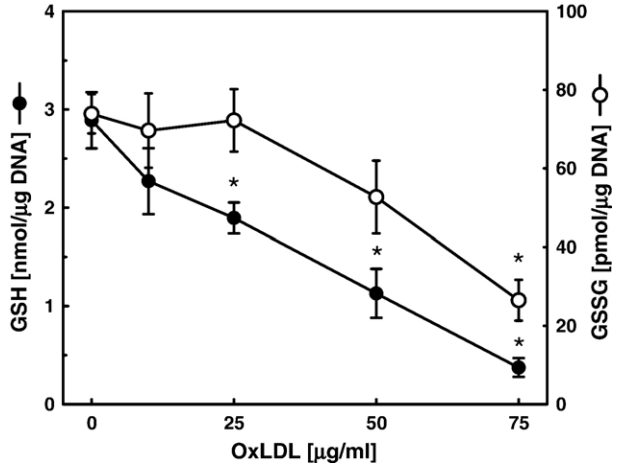

Fig. 2. Effect of OxLDL on GSH and GSSG levels in human macrophages. Human macrophages were incubated with OxLDL at the indicated concentrations. After 24 h, cells were harvested and the cellular total non-protein-bound glutathione (GSH_{tot}) and glutathione disulfide (GSSG, open circles) contents were measured. Cellular GSH levels (closed circles) were calculated as the difference between (GSH_{tot}-2 [GSSG]). GSH and GSSG values are normalized to cellular DNA levels. Results are expressed as mean ± SE of four independent experiments performed in triplicates. * $p < 0.05$ vs no OxLDL added.

GSH stores, selected wells received L-buthionine sulfoximine (BSO), an irreversible inhibitor of γ-glutamyl cystine synthetase, during the 24-h incubation period. DEM treatment (2 h) alone did not significantly decrease macrophage GSH_{tot} levels at 24 h (Fig. 4A, open bar) and had no significant effect on the GSH/GSSG ratio at 24 h (Fig. 4B, open bar), but when DEM-treated cells were incubated in the presence of BSO, GSH_{tot}

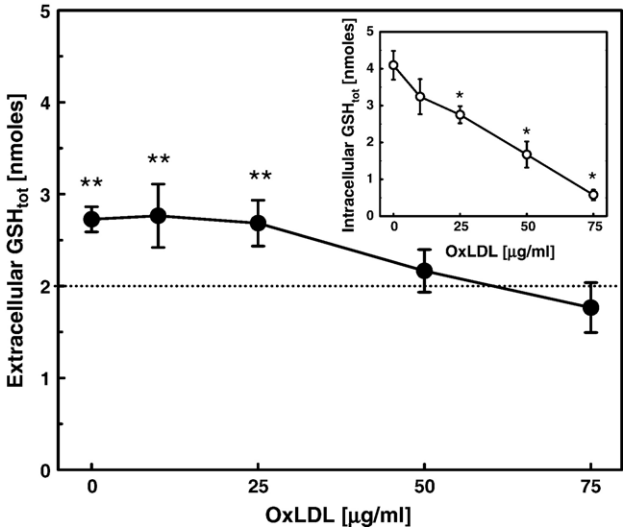

Fig. 3. Effect of OxLDL on GSH and GSSG efflux. Human macrophages were incubated with OxLDL at the indicated concentrations. After 24 h, cells were harvested and total non-protein-bound glutathione (GSH_{tot}) levels were measured in both the macrophages (see insert) and the cell supernatants (Extracellular GSH_{tot}). GSH_{tot} levels were also measured in culture medium incubated for 24 h in the absence of cells (dotted line, 1.97 ± 0.06 µM, 1 ml/well). Extracellular and intracellular GSH_{tot} values are shown as nanomoles per well. Results are expressed as mean ± SE of four independent experiments performed in triplicates. * $p < 0.05$ vs no OxLDL added; ** $p < 0.05$ vs culture medium (no cells present, dotted line).

Table 1
Effects of OxLDL, 4-hydroxynonenal, and diamide on glutathione and glutathione disulfide levels and the GSH/GSSG ratio in human macrophages

	GSH_{tot} [nmol/µg DNA]	GSSG [pmol/µg DNA]	Ratio [mol/mol]
Control	3.03 ± 0.29	73.9 ± 5.0	40.4 ± 4.3
OxLDL (75 µg/ml)	0.43 ± 0.11*	26.5 ± 5.2*	12.6 ± 2.0*
4-HNE (50 µM)	0.26 ± 0.09*	37.8 ± 9.5*	5.3 ± 2.1*
Diamide (0.5 mM)	3.52 ± 1.60	240 ± 31*	15.0 ± 2.4*

Human macrophages were incubated with medium alone (Control), OxLDL (75 µg/ml), 4-hydroxynonenal (4-HNE, 50 µM), or diamide (0.5 mM). After 24 h, cells were harvested and the cellular total non-protein-bound glutathione (GSH_{tot}) and glutathione disulfide (GSSG) contents were measured. Cellular GSH levels were calculated as the difference between (GSH_{tot}-2 [GSSG]). GSH and GSSG values are normalized to cellular DNA levels. Results are expressed as mean ± SE of four independent experiments performed in triplicates.
* $p < 0.05$ vs Control.

levels decreased by 80% (Fig. 4A, open bar). Despite this substantial depletion of GSH, macrophages maintained their GSH/GSSG ratio (Fig. 4B, open bar). This indicates that despite the low concentrations of intracellular GSSG (Fig. 4C, open bar), GR was able to convert sufficient GSSG into GSH to maintain the GSH/GSSG ratio, albeit at a much lower concentration of total GSH. More importantly, we did not detect increased cytotoxicity in GSH-depleted macrophages (Fig. 4D, open bars), indicating that even a major loss of intracellular GSH alone is not sufficient to not promote macrophage death. It should be noted that OxLDL depleted GSH to a similar extent as DEM+BSO (Fig. 4A), yet cytotoxicity was only observed in OxLDL-treated macrophages (Fig. 4D, closed bars).

Trolox prevents neither glutathione depletion nor the collapse of the GSH/GSSG ratio

Previously, we showed that the peroxyl radical scavenger Trolox is a potent inhibitor of OxLDL-induced macrophage death [18]. We therefore examined if Trolox would also restore the glutathione redox state in OxLDL-treated macrophages to that measured in macrophages incubated in the absence of OxLDL (Control). Surprisingly, Trolox, at concentrations that completely prevent macrophage death, did not prevent GSH depletion and did not restore the GSH/GSSG ratio (Fig. 5), demonstrating that a decrease in the GSH/GSSG ratio alone is not sufficient to promote macrophage death. These data confirm that nonoxidative mechanisms, e.g., alkylation, are likely to contribute to the depletion of GSH by OxLDL.

Trolox blocks OxLDL-induced protein-S-glutathionylation

One potential mechanism by which thiol oxidative stress may promote cell injury is through the inactivation of vital metabolic and antioxidant enzymes with catalytically essential, reactive cysteine residues [28,29]. Both, a low GSH/GSSG ratio and an enhanced formation of ROS, e.g., peroxides and peroxyl radicals, are associated with protein-*S*-glutathionylation, the formation of mixed disulfides (PSSG) formed between protein

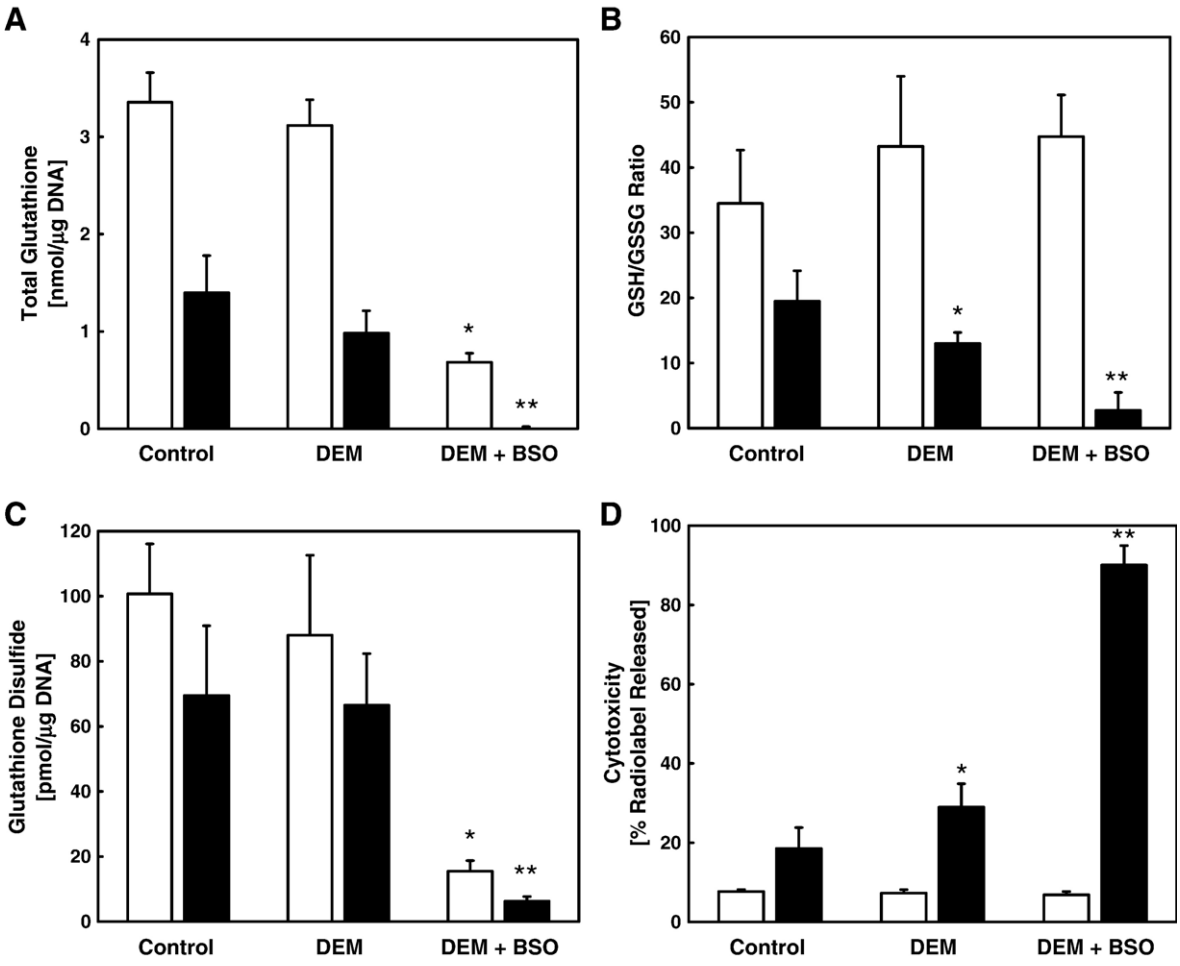

Fig. 4. Effect of GSH depletion on macrophage glutathione redox state and OxLDL cytotoxicity. [^{3}H]Adenine-loaded human macrophages were preincubated for 2 h with 1 mM diethyl maleate (DEM) where indicated, and subsequently stimulated for 24 h either in the absence (open bars) or in the presence of 75 µg/ml OxLDL (closed bars). During cell stimulation, 0.3 mM L-buthionine sulfoximine (BSO) was present where indicated to inhibit de novo GSH synthesis. Values for total non-protein-bound glutathione (A) and GSSG (C) are normalized to cellular DNA levels. For the determination of the GSH/GSSG ratio (B), cellular GSH levels were calculated as the difference between (GSH_{tot}-2 [GSSG]). Macrophage viability (D) was determined as a percentage of radiolabel released from cells. Results are expressed as mean ± SE of four independent experiments performed in triplicates. * $p < 0.05$ vs Control, no OxLDL added (open bars); ** $p < 0.05$ vs OxLDL stimulation in the absence of DEM and BSO (Control, closed bars).

thiols (PSH) and GSH [28]. We therefore examined whether protein-*S*-glutathionylation corresponds to OxLDL toxicity in human macrophages. We found that even in untreated macrophages (Fig. 6A, Control, open bar), the amount of GSH releasable from the protein fraction was significant, and corresponding to approximately 5% of total cellular GSH, i.e., GSH_{tot} plus PSSG. Surprisingly, we observed only a minor, statistically not significant increase in protein-bound glutathione in response to OxLDL treatment (Fig. 6A, Control, closed bar). However, the formation of PSSG is reversible and high *de*glutathionylation activity in the surviving macrophages (≥60%, Fig. 6B) may have masked any measurable accumulation of PSSG in response to OxLDL. Because reduced GSH is required for the regeneration of glutaredoxin, the primary enzyme involved in the deglutathionylation of PSSG [25], we reasoned that inhibition of GR, and thus the conversion of GSSG to GSH, with nonlethal doses of the GR inhibitor BCNU may limit GSH availability sufficiently to impair Grx-mediated deglutathionylation and allow PSSG to accumulate. In the absence of OxLDL, BCNU pretreatment of macrophages for 1 h with 0.2 mM BCNU had no significant effect on PSSG levels, but BCNU-pretreated macrophages showed an 83% increase in PSSG in response to OxLDL stimulation (Fig. 6A, closed bars). This increase in PSSG levels was accompanied by a dramatic increase in macrophage death (Fig. 6B, closed bars), suggesting that protein-*S*-glutathionylation may directly contribute to OxLDL-induced macrophage death. Surprisingly, the peroxyl radical scavenger Trolox not only prevented macrophage death (Fig. 6B), but also completely blocked OxLDL-induced PSSG accumulation (Fig. 6A). This result indicates that peroxides and/or peroxyl radicals may be directly involved in the enhanced *S*-glutathionylation of proteins induced by OxLDL.

Inhibition of glutaredoxin activity enhances OxLDL-induced macrophage death

To further examine whether protein *S*-glutathionylation contributes to OxLDL-induced macrophage death, we generated

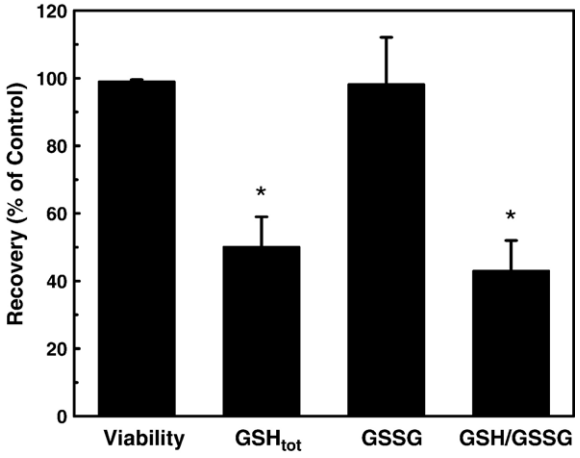

Fig. 5. Effect of Trolox on OxLDL-induced changes in glutathione redox state. [^{3}H]Adenine-loaded human macrophages were stimulated for 24 h either in the absence of OxLDL (100%) or in the presence of 75 μg/ml OxLDL plus 250 μM Trolox, a peroxyl radical scavenger. Cellular GSH and GSSG levels were determined and the GSH/GSSG calculated as described in Fig. 1. Results are expressed as "Recovery," i.e., percentage compared to values for each parameter (cell viability, GSH$_{tot}$, GSSG, GSH/GSSG) obtained in control macrophages incubated for 24 h in the absence OxLDL. Thus, if Trolox had no effect on a measured parameter the recovery would be 0%, whereas if Trolox restored the value of a parameter being measured to the value determined in control macrophages, the recovery would be 100%. * $p < 0.05$ vs no OxLDL added (100%).

siRNA directed against Grx1 and investigated whether inhibition of Grx-mediated deglutathionylation enhances OxLDL cytotoxicity. Transfection of macrophages with anti-Grx1 siRNA decreased Grx activity in macrophages by 75% (not shown), but did not significantly affect macrophage viability (Fig. 7, 0 μg/ml OxLDL). However, knockdown of Grx1 sensitized macrophages to OxLDL cytotoxicity as evidenced by a significant left shift in the dose-response curve (Fig. 7). We observed a similar left shift in the dose-response curve in macrophages transfected with siRNA directed against human GR, the enzyme required for replenishing GSH and thus maintaining Grx in its active state (data not shown). Transfection of macrophages with siRNA directed against luciferase GL2 had no effect on OxLDL cytotoxicity compared to mock-transfected cells (data not shown). These results further support a mechanism by which OxLDL-induced protein-*S*-glutathionylation promotes macrophage death and they suggest that Grx protects macrophages from OxLDL cytotoxicity.

Increasing intracellular GSH levels protects macrophages from OxLDL cytotoxicity

Our results show that GSH depletion alone is not sufficient to promote macrophage death. Yet the Grx data suggest that GSH depletion induced by OxLDL may limit the ability of Grx to prevent the accumulation of PSSG and thus may indirectly contribute to macrophage death. To examine if GSH depletion is required for OxLDL-induced macrophage death, we increased the cellular GSH levels in human macrophages by preincubating the cells for 2 h with 5 mM membrane-permeable glutathione diethyl ester (GDE). GDE, which upon cleavage by cytosolic hydrolases releases GSH, increased macrophage total GSH levels from 3.61 nmol/μg DNA to 5.24 nmol/μg DNA (Fig. 8A, open bars). OxLDL treatment depleted intracellular total GSH by 66% in unsupplemented macrophages but only by 56% in GDE-supplemented cells (Fig. 8A, closed bars), resulting in 86% higher total GSH levels in GDE-supplemented macrophages after OxLDL treatment than in unsupplemented OxLDL-stimulated cells. This difference in GSH was sufficient to diminish OxLDL-induced cytotoxicity by 60% (Fig. 8B), demonstrating that GSH depletion is required for OxLDL to promote macrophage death. Interestingly, we did not observe a

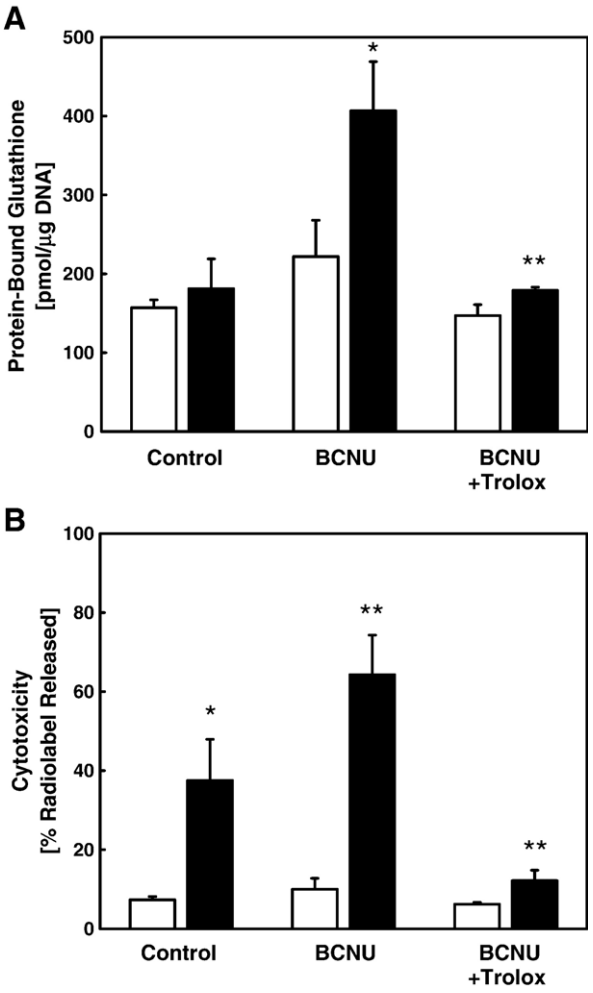

Fig. 6. Effect of OxLDL and inhibition of glutathione reductase on protein-*S*-glutathionylation and macrophage death. [^{3}H]Adenine-loaded human macrophages were stimulated for 24 h either in the absence (open bars) or in the presence of 75 μg/ml OxLDL (closed bars). Where indicated, macrophages were preincubated for 2 h with 0.3 mM 1,3-bis[2-chloroethyl]-1-nitrosourea (BCNU) to inhibit glutathione reductase activity. Protein-*S*-glutathionylation (A) was determined as the amount of GSH released from the macrophage protein fraction after DTT reduction as described under Methods. Cytotoxicity (B) was determined prior to cell harvest as a percentage of radiolabel released from cells. Results are expressed as mean ± SE of four independent experiments performed in triplicates. * $p < 0.05$ vs no OxLDL added (BCNU, open bar); ** $p < 0.05$ vs OxLDL stimulation in the presence of BCNU only (BCNU, solid bar); *** $p < 0.05$ vs OxLDL stimulation in the absence of BCNU (Control, solid bar).

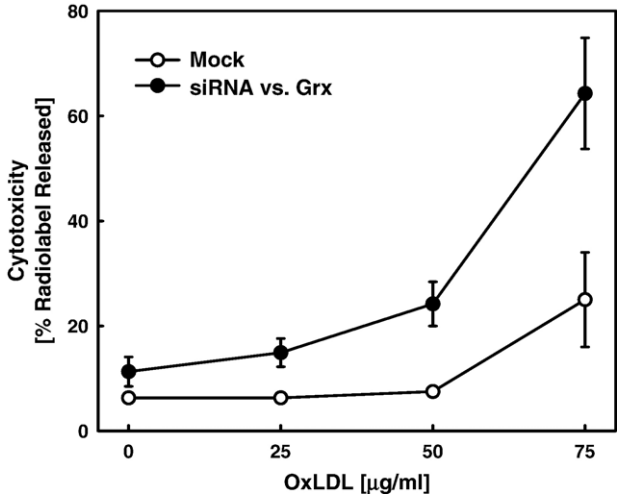

Fig. 7. Effect of siRNA directed against glutaredoxin on OxLDL-induced macrophage death. Macrophages were transfected for 6 h at 37°C either in the absence (Mock, open circles) or in the presence of 0.5 μg siRNA directed against glutaredoxin (closed circles). Cells were washed and incubated for 18 h in culture medium with 5% AB serum. Macrophages were then loaded with [^{3}H] adenine, washed, and stimulated for 24 h with OxLDL at the indicated concentrations. Cytotoxicity was determined as a percentage of radiolabel released from cells. Results are expressed as mean ± SE of four independent experiments performed in triplicates. Data were statistically analyzed using two-way ANOVA ($p = 0.001$ for Mock versus siRNA).

significant improvement of the GSH/GSSG ratio after GDE supplementation (Fig. 8A, numbers in bars).

OxLDL inhibits glutathione reductase activity

GR, the enzyme responsible for replenishing GSH and maintaining Grx activity, is susceptible to inactivation of its catalytically essential Cys-63 residue [30,31]. We therefore explored the possibility that OxLDL further limits GSH availability by inhibiting GR activity and found that GR activity was inhibited by OxLDL in a dose-dependent manner (Fig. 9). In the presence of 75 μg/ml OxLDL, GR activity was reduced by 32% and in the presence of 100 μg/ml OxLDL by 44%. The OxLDL-induced decrease in GR activity was completely prevented by Trolox (Fig. 9, open circle), indicating that peroxyl radicals are involved in the inactivation of GR. Together, these data further support a critical role for GSH availability in macrophage death and suggest that maintaining GR activity is essential for the protection of macrophages against OxLDL cytotoxicity.

Discussion

The glutathione-dependent antioxidant system plays a major role in protecting cells from dysfunction and cell injury. The present study shows that in human macrophages OxLDL promotes thiol oxidative stress and cell injury by disrupting the GSH redox state. Three major pathways appear to contribute to this process: (1) depletion of GSH, (2) inhibition of GR, and (3) oxidation of protein thiols (Fig. 10). Furthermore, our data suggest that the convergence of these three pathways results in enhanced protein-*S*-glutathionylation, and that the ensuing loss of protein function(s) leads to macrophage death.

GSH depletion is required for OxLDL-induced macrophage toxicity (Fig. 10). This conclusion is based on the observation that increasing intracellular GSH levels via GDE supplementation (partially) protected against OxLDL-induced macrophage death. Conversely, pharmacological depletion of intracellular GSH dramatically increased the susceptibility of macrophages to OxLDL-induced killing. However, GSH depletion, even by as much as 80%, by itself does not promote macrophage death, indicating that the OxLDL-induced decrease in intracellular GSH is necessary, but not sufficient to induce macrophage injury. This conclusion is further supported by the observation

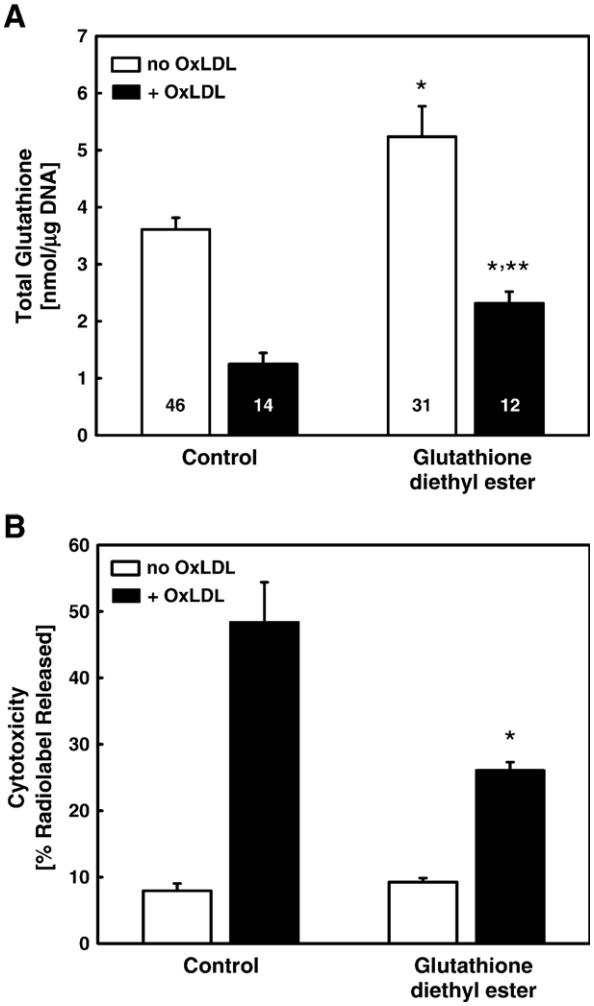

Fig. 8. Effect of glutathione diethyl ester on OxLDL-induced macrophage death. [^{3}H]Adenine-loaded human macrophages were preincubated for 2 h either in the absence (Control) or presence of 5 mM glutathione diethyl ester (GDE), a cell-permeable precursor of GSH, and then stimulated with either culture medium alone (open bars) or OxLDL (closed bars). After 24 h, cells were harvested and intracellular total non-protein-bound glutathione (A) and macrophage viability (B) values were measured as described under Methods. Values for total non-protein-bound glutathione (GSH_{tot}) are normalized to cellular DNA levels. The numbers in the bars (A) are the estimated mean values for the cellular GSH/GSSG ratios for the respective condition. Cellular GSH levels were calculated as the difference between (GSH_{tot}-2 [GSSG]). * $p < 0.05$ vs no GDE added; ** $p < 0.05$ vs no OxLDL added.

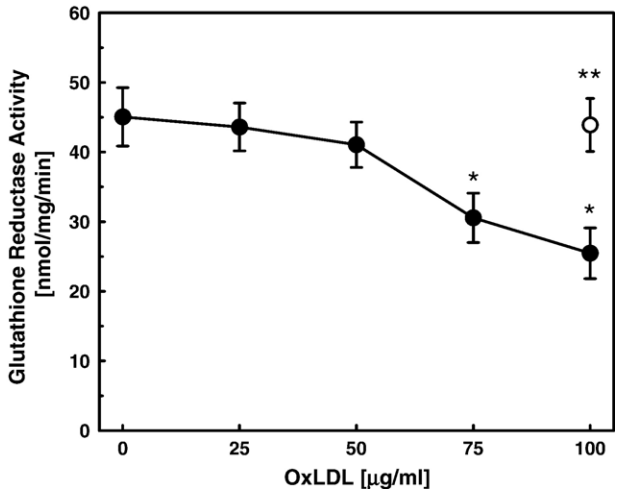

Fig. 9. Effect of OxLDL on glutathione reductase activity in human macrophages. Human macrophages were stimulated for 24 h with OxLDL at the indicated concentrations, either in the absence (closed circles) or in the presence of 250 μM Trolox (open circle). After 24 h, cells were harvested and the cellular glutathione reductase activity was measured as described under Methods. * $p < 0.05$ vs no OxLDL added, ** $p < 0.05$ vs 100 μg/ml OxLDL.

that the peroxyl radical scavenger Trolox completely blocked OxLDL cytotoxicity and OxLDL-induced PSSG formation, but did not restore bulk GSH levels. Our findings are in good agreement with results obtained in monocytic THP-1 cells by Darley-Usmar and colleagues, demonstrating that depletion of cellular GSH also enhances OxLDL cytotoxicity in macrophage-like cell lines [16].

Interestingly, both undifferentiated and PMA-differentiated THP-1 cells respond to OxLDL exposure with increased GSH synthesis, resulting in a more than 2-fold increase in total intracellular GSH [16,17]. Increased expression of enzymes involved in GSH synthesis in response to OxLDL was also observed in the murine macrophage-like cell lines Raw 264.7 and J774 A.1 as well as mouse peritoneal macrophages [14,15]. However, in these latter studies actual changes in intracellular GSH levels were not reported. In contrast to these immortalized monocytic cell lines, we did not detect an increase in total glutathione levels in primary monocyte-derived macrophages in response to any OxLDL concentration tested. While we cannot rule out that OxLDL stimulated GSH synthesis in human macrophages, any increase in GSH synthesis induced by OxLDL clearly was insufficient to compensate for the GSH-depleting effect of OxLDL. This apparent inability of human monocyte-derived macrophages to respond to thiol oxidative stress with increased GSH synthesis distinguishes primary (human) macrophages from the widely used immortalized and highly proliferative macrophage cell lines and may also explain why in contrast to GDE, cell supplementation with 10 mM *N*-acetylcysteine, a membrane-permeable form of the GSH precursor cysteine, did not protect from OxLDL cytotoxicity (unpublished data).

How OxLDL depletes cellular GSH in macrophages is not clear. The degradation of GSH catalyzed by γ-glutamyl transpeptidase occurs extracellularly [32]. Therefore, only two mechanisms are likely to have contributed to the depletion of cellular GSH: (1) GSH (or GSSG) efflux or (2) DTT-resistant modifications of GSH, e.g., *S*-alkylation. Although we detected a significant release of GSH (or GSSG) by human macrophages, GSH efflux did not account for the OxLDL-induced depletion of GSH, suggesting that GSH conjugation may have contributed to the loss of GSH. The oxidation of LDL generates lipid (hydro)peroxides which upon decomposition form reactive aldehydes [27]. Reactive aldehydes, including 4-hydroxy alkenals, readily undergo Michael additions with GSH, and the resulting conjugates are eliminated by GSH-*S*-transferase-mediated pathways. It is therefore possible that OxLDL-derived aldehydes generated during LDL (per)oxidation are responsible for the depletion of GSH. However, if reactive aldehydes generated during LDL oxidation, i.e., prior to the interaction of OxLDL with macrophages, were capable of depleting millimolar concentrations of intracellular GSH, we would not expect to find significant levels of GSH in culture media exposed to OxLDL. Yet, we consistently detected micromolar concentrations of glutathione in cell supernatants of OxLDL-treated macrophages (Fig. 3). Hardwick and colleagues reported that supplementation of culture medium with 5 mM GSH protected

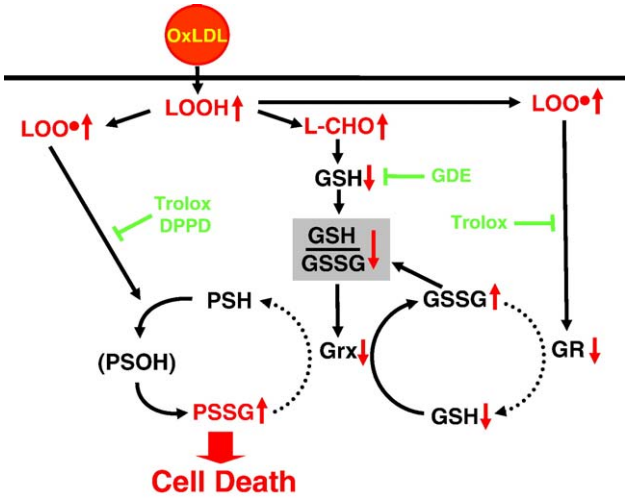

Fig. 10. Proposed model for the role of the glutathione-dependent antioxidant system in OxLDL-induced macrophage death. OxLDL-derived lipid (hydro) peroxides (LOOH) are transferred from the lipoprotein particle into the macrophage. The decomposition of LOOH yields intracellular peroxyl radicals ($LOO^{\bullet}$) and reactive aldehydes (L-CHO). In a $LOO^{\bullet}$-dependent pathway, LOOH promote the oxidation of protein thiols (PSH) to the corresponding sulfenates (PSOH), which in the presence of GSH form mixed disulfides (PSSG). Peroxyl radical scavengers Trolox and DPPD prevent PSSG accumulation and macrophage death. In a second, $LOO^{\bullet}$-independent pathway, OxLDL promotes the depletion of GSH and in a third, $LOO^{\bullet}$-dependent pathway, OxLDL inhibits glutathione reductase activity (GR), possibly by modifying the critical active site Cys residue. Loss of GR activity enhances the accumulation of glutathione disulfide (GSSG), which together with the depletion of GSH results in the disruption of the GSH/GSSG ratio. Decreased availability of GSH and a reduced GSH/GSSG ratio are likely to impair glutaredoxin (Grx) activity and thus the deglutathionylation of PSSG and restoration of PSH. Enhanced PSH oxidation combined with decreased protein-*S*-deglutathionylation promotes the accumulation of PSSG, loss of protein function, and subsequent macrophage death. Supplementing cellular GSH stores by treating cells with the cell-permeable GSH precursor GDE protects macrophages from OxLDL-induced cell death.

against OxLDL cytotoxicity in human monocyte-derived macrophages [33]. The authors proposed that extracellular GSH reacts with 4-hydroxynonenal found in OxLDL, thereby detoxifying the lipoprotein. We found that addition of 4-hydroxynonenal to human macrophages depletes intracellular GSH (Table 1), and we could confirm that 4-hydroxynonenal induces cell death in human macrophages. However, in contrast to OxLDL, 4-hydroxynonenal-induced macrophage death was not prevented by Trolox (unpublished data), suggesting that the more water-soluble, short-chain reactive aldehydes are not likely to account for OxLDL cytotoxicity nor the GSH-depleting effect of OxLDL. Together, these data suggest that neither GSH depletion nor macrophage death induced by OxLDL are mediated by OxLDL-derived aldehydes that were generated during LDL oxidation, i.e., prior to the interaction of OxLDL with macrophages.

We propose an alternative mechanism, in which OxLDL-derived lipid (hydro)peroxides (LOOH) are transferred into human macrophages, where they decompose to form peroxyl radicals ($LOO^{\bullet}$) and reactive aldehydes (L-CHO, Fig. 10). According to this model, the actual thiol-oxidizing and GSH-depleting compounds are not generated during LDL oxidation, but rather in situ, i.e., after the interaction of OxLDL with macrophages. Recent studies from our laboratory show that uptake of OxLDL may not be required to induce macrophage death, suggesting that the LOOH transfer may occur by diffusion [34]. Our hypothetical mechanism is in good agreement with a model previously proposed by Chisolm and colleagues for the cytotoxicity of OxLDL in endothelial cells [35]. Like in Chisolm's model, we would predict that LOOH decomposition is catalyzed by intracellular heavy metal ions, possibly by Fe^{3+}. The Fe^{3+}-catalyzed breakdown of LOOH generates $LOO^{\bullet}$ whereas in the presence Fe^{2+} ions, LOOH yields primarily alkoxyl radicals ($LO^{\bullet}$) [36], which, as we showed previously, do not appear to be involved in OxLDL-induced macrophage death [18]. The existence of an intracellular pool of accessible, redox-active iron, often referred to as labile iron pools (LIP), is now well-recognized [37] and LIP have been reported both in primary macrophages and in monocytic cell lines [38,39].

Furthermore, we would predict that increased peroxyl radical formation ($LOO^{\bullet}$, Fig. 10) will result in increased protein thiol oxidation, formation of the corresponding, highly reactive sulfenates, and the subsequent formation of PSSG (Fig. 10). If the GSH/GSSG redox state is intact, macrophage Grx activity would prevent any lethal accumulation of PSSG. However, in addition to $LOO^{\bullet}$, LOOH decomposition is also likely to yield reactive aldehydes, including alkenals. These aldehydes would react with intracellular GSH, resulting in the observed depletion of GSH and the collapse of the GSH/GSSG ratio. We would predict that this change to a more oxidizing thiol redox environment would sufficiently impair Grx-mediated deglutathionylation to allow PSSG to accumulate to a lethal level. Our siRNA knockdown experiments demonstrated that impaired Grx activity dramatically increased the sensitivity of macrophages to OxLDL cytotoxicity. Yet in the presence of Trolox, cell viability was completely restored in Grx-deficient macrophages treated with OxLDL. This result strongly suggests that both reduced protein deglutathionylation and enhanced protein thiol oxidation (by peroxyl radicals) are required for OxLDL to promote macrophage death (Fig. 10).

Depletion of cellular GSH with DEM plus BSO was not sufficient to promote macrophage death. A likely explanation for this observation is that in contrast to OxLDL, DEM plus BSO treatment did not significantly alter the GSH/GSSG ratio, i.e., the thiol redox environment (Fig. 4B). The GSH/GSSG ratio is maintained by GR and both BCNU- and siRNA-mediated inhibition of GR increased OxLDL cytotoxicity. Therefore, the partial inhibition of GR activity induced by OxLDL in addition to its GSH depletive effect may explain why OxLDL has such a dramatic effect on the GSH/GSSG ratio in macrophages.

In summary, our data suggest that OxLDL promotes peroxyl radical formation and GSH depletion by two concurrent pathways, potentially triggered by the transfer of lipid (hydro) peroxides from OxLDL into macrophages. Neither pathway is sufficient to induce cell injury, but when stimulated concurrently, these pathways promoted protein-*S*-glutathionylation and macrophage death. Our results demonstrate further that the glutathione/glutaredoxin system plays a critical role in protecting macrophages from OxLDL-induced cell death and that compromising this system renders macrophages susceptible to oxidative stress-induced cell injury. Because the glutathione/glutaredoxin system is intimately involved in the recycling of dietary antioxidants such as vitamins E and C, a compromised glutathione/glutaredoxin system in macrophages and foam cells in atherosclerotic lesions may at least in part explain why classic antioxidant therapy by and large has not proven successful in combating atherosclerosis.

Acknowledgments

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