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THE THIOL REDOX SYSTEM IN OXLDL-INDUCED MACROPHAGE INJURY

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ABSTRACT OF THESIS

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Macrophage death is likely to contribute to the transformation of fatty streaks into advanced atherosclerotic lesions. Previous work in the laboratory showed that OxLDL promotes cell death in human macrophages by a mechanism involving intracellular peroxide formation. Here we show that glutathione depletion induced by OxLDL occurs independent of peroxyl radical formation. Our data suggest that the depletion of glutathione is the fundamental defect that renders macrophages susceptible to OxLDL-induced cell injury, but alone is not sufficient to kill macrophages. We indicate that increased protein-*S*-glutathionylation is involved in OxLDL-induced macrophage death. A potentiation of OxLDL toxicity was observed in macrophages transfected with siRNA directed against either glutathione reductase or glutaredoxin. Our data suggests that OxLDL-induced cell injury in human macrophage is mediated by the depletion of GSH, a decreased in the GSH/GSSG ratio and peroxyl radical formation. All three signals are required for OxLDL-induced macrophage death. Our results also show that the glutathione reductase/glutaredoxin system protects macrophages from OxLDL-induced cell death.

KEYWORDS: Macrophages, Oxidized LDL (OxLDL), Glutathione (GSH), Glutaredoxin (GRx), Glutathione Reductase (GR)

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Yanmei Wang

The Graduate School
University of Kentucky
September, 2005

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THESIS

A thesis submitted in partial fulfillment of the requirements
for the degree of Master of Science in Nutritional Sciences at
the University of Kentucky

By
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CHAPTER ONE

INTRODUCTION

Background

Atherosclerosis is a disorder of lipid metabolism as well as a chronic inflammatory disease. Complications of atherosclerosis are the most common causes of death in western countries. The molecular mechanisms for pathogenesis of atherosclerosis have been widely investigated. Recent studies indicate that atherosclerosis can be considered a form of chronic inflammation resulting from interaction between modified lipoproteins, monocyte-derived macrophages, T cells, and the normal cellular elements of the arterial wall such as free cholesterol, cholesterol ester, triglyceride, phospholipids, and calcium salt content of the arteries. [1].

Atherosclerosis lesions begin as fatty streaks underlying the endothelium of large arteries. A large number of studies have implicated oxidatively modified LDL (OxLDL), macrophage dysfunction and cell death in the transformation of fatty streaks into advanced atherosclerotic lesions [2, 3]. Oxidation of LDL is induced by reactive oxygen species (ROS) produced by endothelial cells [4], vascular smooth muscle cells [5], or monocytes [6]. According to the “LDL retention hypothesis” by Williams and Tabas [7], LDL initially accumulates in the extracellular subendothelial space of arteries and, is mildly oxidized to minimally modified LDL. Minimally modified LDL promotes the migration of monocytes into the subendothelial space and their conversion into macrophages [2]. The increased ROS in the microenvironment convert the mildly oxidized LDL into highly oxidized LDL (OxLDL). OxLDL promotes lipoprotein aggregation and foam cell formation [8-10]. The subsequent elaboration of growth factors, secretion of other potent biological factors by the monocyte-macrophages, and death of the foam cells would result in progression of the atherosclerosis lesion [1, 2, 7]. OxLDL aggregation enhances OxLDL uptake and metabolism by human macrophages, but decreases OxLDL-induced cell injury, suggesting that OxLDL

aggregation may reduce vascular cell injury and necrotic core formation [11] (Figure 1). Taken together, oxidation of LDL and macrophage dysfunction and cell death play key roles in the formation and progression of atherosclerosis.

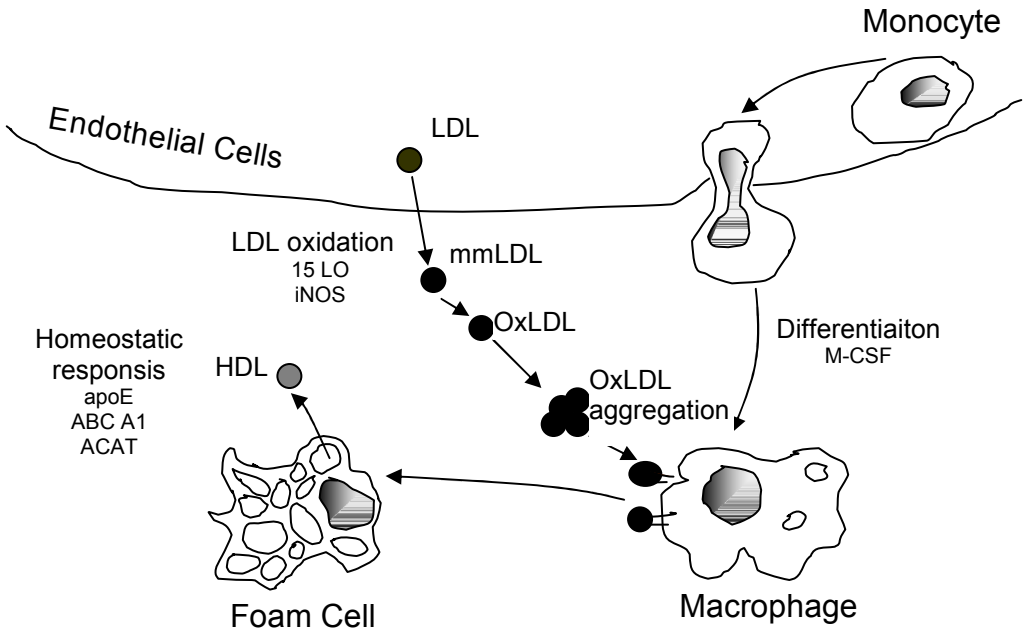

Figure 1 Monocyte recruitment and foam cell formation. (Adapted from Glass et al. Cell. 2001 104(4):503-16, Ref. 1)

The functional roles of OxLDL

In the late 1980s, Steinberg and other scientists demonstrated that the cells of the artery wall are constantly secreting ROS into their membranes and the subendothelial space [12-15]. Phospholipids contained in LDL that become trapped in the artery, are oxidized as a result of exposure to ROS released from artery wall cells. OxLDL stimulates monocytes to attach to the endothelial cells, migrate into the subendothelial space and differentiate into macrophages [1, 2, 7]. Thus, monocyte-derived macrophages take up OxLDL via scavenger receptors

and OxLDL receptors such as SR-A and CD36, leading to foam cell and fatty streak formation which are important events in atherogenesis [3, 16, 17].

Oxidation of LDL renders it cytotoxic for all cells involved in atherogenesis, including endothelial cells [18], lymphocytes [19], and monocyte-derived macrophages [20]. But the complex mechanism by which OxLDL induces cell injury is mostly unknown and the components of OxLDL which mediate cell injury are not clear. OxLDL contains high concentrations of lipid peroxidation products such as peroxides and aldehydes [21, 22]. Recent data from our lab have shown that OxLDL promotes peroxide formation and OxLDL-derived lipid (hydro)peroxides are responsible for the cytotoxicity of OxLDL in human macrophages [23]. Coffey and coworkers demonstrated that in fibroblasts, OxLDL cytotoxicity involves lipid peroxidation and iron-mediated radical formation [24]. Esterbauer and coworkers have shown that aldehydes, breakdown products of lipid peroxidation, are present in OxLDL particle and are important determinants of OxLDL toxicity [22]. OxLDL has been shown to cause an initial decrease followed by an adaptive increase of GSH in the human monocyte cell line THP-1 and macrophages isolated from C3H/HEJ mice [25]. In addition, OxLDL promotes lysosomal damage in J-774 cells [26]. Hence, OxLDL-induced macrophage death may involve ROS formation, oxidative stress/antioxidant imbalance, alteration of thiol redox state, organelle damage, and cell lysis.

Synthesis and functional roles of glutathione (GSH)

GSH is the predominant low molecular thiol in mammalian cells and is known to function in many important biological phenomena, including the synthesis of proteins and DNA, transport, enzyme activity, metabolism, and protection of cells [27]. The majority of GSH in cells is found in the cytosol at a concentration of 1-11 mM [28]. Cellular GSH levels reflect a steady state balance between GSH synthesis and combined rate of GSH consumption within the cell and loss through efflux or GSH conjugation. The cytosol appears to be the principal location of GSH *de novo* biosynthesis. GSH is synthesized from L-

glutamate via two ATP-dependent enzymatic reactions that are catalyzed by γ-glutamylcysteine synthetase (γ-GCS) and glutathione synthetase (GS) (Figure 2) [27, 29, 30]. GSH transported across cell membranes can be extracellularly converted to γ–Glutamyl amino acids via the catalysis of γ-glutamyl transpeptides. γ–Glutamyl amino acids are taken up by cells and act as substrates for GSH *de novo* synthesis. GSH also can be regenerated from GSSG by glutathione reductase (GR) [30-32]. Various drugs have been developed to modulate GSH level in cells and tissues. For example, L-buthionine-sulfoximine (BSO) selectively inhibits γ-glutamylcysteine synthetase (γ-GCS), thereby inhibiting the synthesis of γ-glutamylcysteine, a precursor of glutathione. BSO inhibits GSH

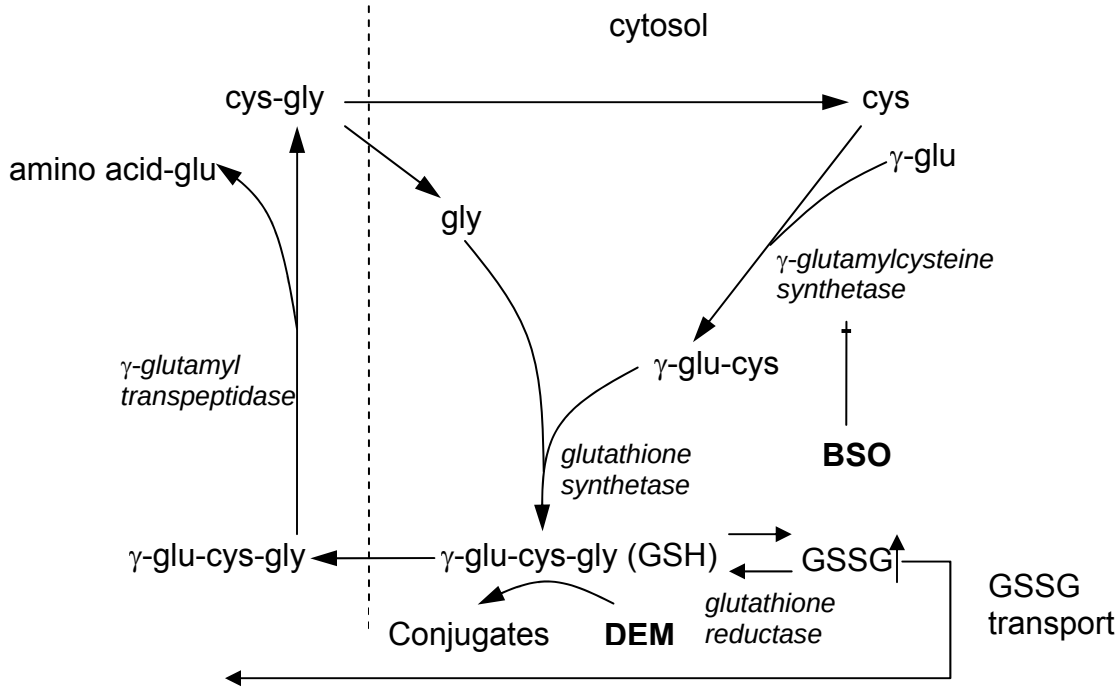

Figure 2 Glutathione synthesis and salvage pathways. (Adapted from A.G.Hall. European Journal of Clinical Investigation. 1999. 29(3):238-45, Ref. 30)

biosynthesis and causes depletion of cellular GSH levels [33-36]. Although cytosolic GSH synthesis is blocked, the cell's use of GSH, the export of oxidized glutathione (GSSG), and the reduction of GSSG by glutathione reductase continue. GSH can also be depleted by the alkylating agent diethyl maleamide (DEM). Unlike BSO, DEM combines with free thiols to prevent the use of GSH, but not its synthesis [34].

GSH can directly scavenge free radicals. For example, GSH reacts rapidly and nonenzymatically with hydroxyl radical, the cytotoxic Fenton reaction product, and with N_2O_3 and peroxynitrite, cytotoxic products formed by the reaction of nitric oxide with O_2, respectively [37-40]. But it does not easily react with other more common ROS such as superoxide and H_2O_2 [41]. GSH also acts as a substrate for glutathione peroxidase (GPx), glutathione-*S*-transferase (GST) and glutaredoxin (GRx) during the detoxification of hydrogen peroxide (H_2O_2), lipid hydroperoxides, electrophilic compounds and *S*-glutathionylated proteins [31, 37]. Each of these reactions leads directly or indirectly to the formation of glutathione disulfide (GSSG), a species that is reduced intracellularly to GSH by glutathione reductase (GR) in a NADPH-dependent reaction. At normal levels of oxidative and nitrosative stress, GR activity and NADPH availability are sufficient to maintain [GSH]/[GSSG] > 100 in the cytosol in most cells and tissues. But under pathophysiological conditions such as cardiovascular and neurodegenerative diseases, the oxidative stress levels increase sufficiently or other factors limit the reaction of GR to induce an increase of GSSG, GSSG may accumulate and the redox balance can shift toward an oxidizing milieu [42]. This has two important consequences: i) the thiol redox status of the cell will shift, activating certain oxidant response transcriptional elements such as protein-1 transcription factor (AP-1) and nuclear factor kappa B (NFκB) [43, 44] and ii) GSSG may be preferentially secreted from the cell. The shift in the ratio of GSH/GSSG would change the redox state to a more positive potential. Export of GSSG would prevent this shift and protect cells and tissues from oxidative stress. Two GSSG transport systems, ATP-driven and carrier-mediated mechanisms have been proven to regulate the GSSG transport [45, 46]. Because GSSG is not taken up

by cells, but is rather degraded extracellularly, loss of GSSG from cells under conditions of oxidative stress increases cellular requirements for *de novo* GSH synthesis [47].

Reversible protein *S*-glutathionylation

Numerous proteins contain sulfhydryl groups (PSH) due to their cysteine content. In fact, the concentration of PSH groups in cells and tissues is much greater than that of GSH [48, 49]. A crucial component of the cellular redox balance is modulation of the thiol disulfide status of critical cysteine residues on proteins. Reactive oxygen/nitrogen species (ROS/RNS) induce cysteine oxidation upon reaction between the protein thiol groups and low-molecular-mass thiols (*S*-thionylation), particularly with GSH (*S*-glutathionylation) to produce protein-mixed disulfide (PSSG). GSH is the most abundant low-molecular-mass thiol in mammalian cell and is the dominant ligand in this reaction. Removal of the thiol adduct through chemical or enzymatic reduction of *S*-glutathionylated protein is defined as *de*glutathionylation. Glutathionylation/*de*glutathionylation reactions have two major functions: i) modification of the functional capacity of the protein and ii) Protection against irreversible changes caused by oxidative modification. In many reports, *S*-glutathionylation is characterized as an inhibitory modification whereby *de*glutathionylation reverses the modification and restores activity [50]. Examples are protein tyrosine phosphatase 1B (PTP1B) [51, 52] and nuclear factor kappa B (NF-κB) [53]. However, in a number of cases *S*-glutathionylation activates proteins, e.g., HIV-1 protease, microsomal glutathione *S*-transferase [54, 55]. Irreversible modifications induced by oxidative stress are generally associated with permanent loss of protein function and may lead to either the progressive accumulation of the damaged proteins, as observed in age-related neurodegenerative disorders or their degradation. It is also hypothesized that cells use glutathionylation to protect the protein thiols from protein-protein disulfide crosslinking or other forms of permanent oxidative damage during oxidative stress until a repair enzyme system can de-thiolate the

PSSG and restore the original structure and function of the protein [55]. For example, aldehydes derived from the peroxidation of polyunsaturated fatty acids, may covalently react with functional groups of the IkB kinase (IKK), the kinase responsible for the activation of NFκB, thereby causing irreversible loss of IKK function and dysregulation of signaling pathways involved in activation of NFκB [56, 57]. However, reversible *S*-glutathionylation of IKK acts as a cellular regulatory mechanism of signal transduction [58].

Accumulations of protein-SSG have been reported in different cell types including myocytes, macrophages, and hepatocytes when they are exposed to t-butyl hydroperoxide, menadione, or diamide to model the effects of oxidative stress [59-61]. Protein *S*-glutathionylation/*de*glutathionylation is a dynamic process that occurs under physiological conditions in cells. Increased levels of PSSG also can occur under physiologic circumstances, during the respiratory burst in human neutrophils [62]. Protein *S*-glutathionylation/*de*glutathionylation status depends on the redox state of the GSH-system in the cell [63]. Identification of specific mechanism for PSSG formation remains under active investigation. Several mechanisms have been proposed for PSSG formation:

(1) $PS^{\bullet} + GSH \rightarrow PSSG + O_2^{\bullet-} + H^+$

(2) $PSOH + GSH \rightarrow PSSG + H_2O$

(3) $PSH + GSSG \leftrightarrow PSSG + GSH$

Mechanism 1: Reactive oxygen species (ROS) as inducers of *S*-glutathionylation [reaction (1) and (2)]. Direct oxidation of protein cysteine by ROS generates a reactive protein thiol intermediate such as a thiyl radical ($PS^{\bullet}$) or sulfenic acid (PSOH), which further reacts with GSH to form the mixed disulfide [64]. This mechanism derives from the intriguing observation that *S*-glutathionylation may occur in intact cells in various experimental models of ROS generation without any detectable changes in the GSH/GSSG ratio [65, 66].

Mechanism 2: Redox-dependent *S*-glutathionylation [reaction (3)]. Mixed disulfide formation occurs in response to changes in the GSH/GSSG ratio through a thiol-disulfide exchange mechanism. In models of oxidative stress, transient shifts in the GSH/GSSG ratio from 100 to 10 even 1 have been

described and found to correlate with the amount of protein mixed disulfide formation [67-69]. For example, a pronounced change in the GSH/GSSG was observed recently in an erythrocyte model of oxidative stress-induced haemoglobin *S*-glutathionylation [70]. But this mechanism is not favored by many people. Large shifts in the intracellular redox potential are believed to be unlikely to occur under normal physiological condition [49] because of the capability of cells to efflux of GSSG as protective mechanism against oxidative stress [71].

Glutaredoxin (GRx) and glutathione reductase (GR) system

An abundance of data from biochemical studies supports *S*-glutathionylation/*de*glutathionylation as a homeostatic mechanism for protecting and restoring function of proteins altered by oxidative conditions. Proteins undergo *S*-glutathionylation under conditions of oxidative stress as described above. Reversal of *S*-glutathionylation (*de*thiolation or *de*glutathionylation) could be achieved by either changes in the intracellular redox status such as increases in the GSH/GSSG ratio, reduced thiols, or via enzymatic reduction by members of the thiol-disulfide oxidoreductase (TDOR) family including the thioredoxin (TRx) system and glutaredoxin (GRx) system [63, 72]. GRx is a 12-kDa cytosolic protein that has been characterized *in vitro* as a specific catalyst for the reduction of protein-glutathionyl-mixed disulfides (PSSG) [73-75]. Likewise, TRx has also been shown to specifically catalyze the reduction of inter- or intra-protein-protein disulfides and sulfenic acid formation of cysteine moieties of proteins [55, 76]. In particular, Mieyal and coworkers found that GRx is specific for PSSG, and the catalytic intermediate GRx-SSG is selectively recycled to the reduced enzyme by GSH, with formation of GSSG and regeneration of GSH by coupling with NADPH and glutathione reductase (GR) [42, 77] (Figure 3). These characteristic interactions of GRx with GSH distinguish it from TRx, which favors intramolecular disulfide substrates and is turned over by NADPH and thioredoxin reductase (TRxR), independently of GSH [76, 78]. GRx enzyme has served as a focal point and important tool for evolution of mechanism of reversible *S*-glutathionylation

because of its characterization as a specific and efficient catalyst of *de*glutathionylation of PSSG. Hence, much attention has been paid to the *de*glutathionylation of GRx. Recently some interesting findings characterized GRx as a versatile catalyst, capable of facilitating *S*-glutathionylation. For example, it has been shown that GRx can mediate *S*-glutathionylation of protein tyrosine phosphatase 1B (PTP1B) under certain conditions such as H_2O_2 treatment [77, 79].

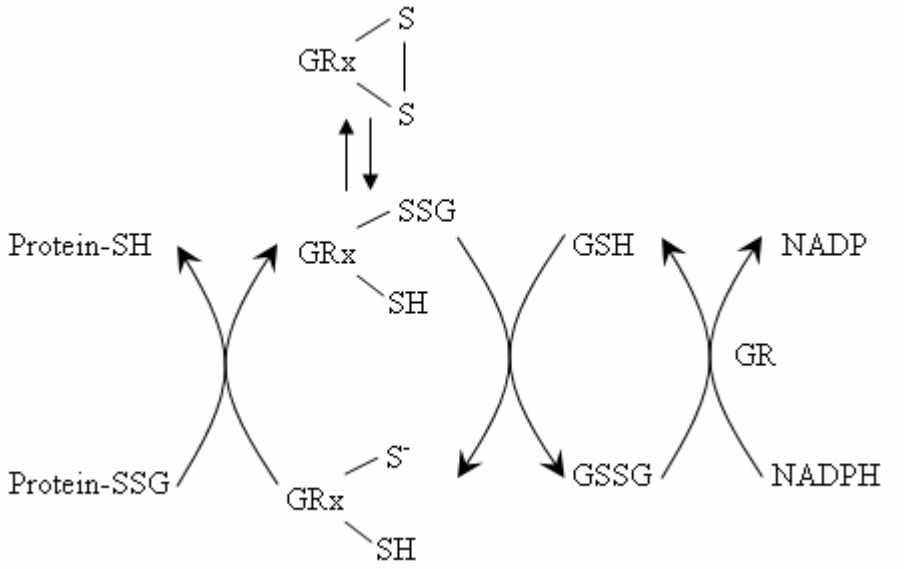

Figure 3. General mechanism of the glutaredoxin system. (Adapted from Melissa D. Shelton et al. Antioxidants and Redox Signaling. 2005.7(3-4):348-66, Ref.77)

Hypothesis and objectives of the present study

Macrophage death is likely to contribute to the transformation of fatty streaks into advanced atherosclerotic lesions. A large number of studies have implicated oxidatively modified LDL (OxLDL) in macrophage and foam cell death, but the underlying mechanism is not fully understood. Previously, our lab showed that OxLDL promotes cell death in human macrophages by a caspase-3 independent mechanism involving intracellular peroxide formation, mitochondrial dysfunction and subsequent loss of plasma membrane integrity. The oxidation of

LDL produces a complex mixture of lipid hydroperoxides, oxysterols, and aldehydes [80-82]. Asmis et al. pointed out that the precursors of peroxyl radicals are most likely lipid hydroperoxides, particular 7-hydroperoxyl cholesterol [23]. There are two possible ways by which hydroperoxides enter the cell: i) Uptake of the entire OxLDL particle or ii) selective uptake of only the lipid hydriperoxides [83]. However, Asmis et al. demonstrated that peroxyl radical formation alone cannot explain OxLDL-induced macrophage death [23]. In primary human cells, including human macrophages, vitamin C (ascorbic acid) and glutathione are the principal free radical scavengers [84]. Their intracellular concentrations are typically between 1 – 10 mM. In culture, however, vitamin C is rapidly depleted from human monocyte-derived macrophages, leaving glutathione as the only major antioxidant in the aqueous compartments. Because OxLDL promotes sustained intracellular oxidative stress in macrophages [84], it is expected that glutathione redox state to be compromised in cells exposed to OxLDL. *In vitro*, exposure of the monocyte cell line THP-1 and macrophages isolated from C3H/HEJ mice to OxLDL results in an initial depletion of GSH [25]. The components of OxLDL which are mediating the decrease of GSH are unclear. Since 4-HNE, an aldehyde, can elicit a similar response, it is likely that 4-HNE or other aldehydes in the OxLDL particle contribute to OxLDL-induced macrophage death [25]. We therefore hypothesized that 1) in addition to peroxide formation OxLDL-induced macrophage death involves change in the glutathione redox state induced by 4-HNE or other aldehydes.

Alterations in the celluar redox state can promote protein-*S*-glutathionylation (PSSG). Accumulation of PSSG also has been reported in different cell types under oxidative stress such as H_2O_2 [66, 85, 86]. Significant increases in PSSG have been found in human diseases such as hyperlipidemia [87], chronic renal failure [88], and diabetes mellitus [87-90]. We therefore hypothesized further that 2) OxLDL promotes the accumulation of PSSG that may contribute to OxLDL-induced macrophage death.

Since GRx enzyme is the primary intracellular catalyst of *de*glutathionylation of PSSG [73-75]. GR and GSH are required for its

regeneration [42]. We finally hypothesized that 3) inhibition of the Grx and/or GR sensitizes human macrophages to OxLDL-induced cell injury.

The goal of this study was to investigate whether OxLDL alters the thiol redox state of human macrophages, whether changes in the thiol redox state are both necessary and sufficient to promote macrophage death, and to determine whether the glutathione reductase/glutaredoxin system plays a key role in protecting macrophages from OxLDL-induced cell injury.

CHAPTER TWO

MATERIALS AND METHODS

Chemicals

1,3-bis[2-Chloroethyl]-1-nitrosourea (BCNU), L-Buthionine-[S,R]-Sulfoximine (BSO), Triton X-100, Diethyl maleate (DEM), Dithiothreitol (DTT), N-ethyimaleimide (NEM), *o*-phthalaldehyde (OPA), reduced glutathione, 6-Hydroxy-2,5,7,8-tetremethyl-chroman-2-carboxylic acid (Trolox), HEPES, Tris-HCl, Sodium Phosphate, Potassium Phosphate, NaCl and KCl were purchased from Sigma-Aldrich-Fluka (St. Louis, MO). Acetonitrile, Methanol and Propionic Acid were purchased from Fisher Scientific (Pittsburgh, PA). [^{3}H]-adenine was supplied by Amersham Biosciences Corp. (Piscataway, NJ). Culture medium (RPMI), GLUTAMAX-1, 1% v/v nonessential amino acids, 1 mmol/L Sodium Pyruvate were purchased from Gibco BRL (Carlsbad, CA). Aprotinin, Leupeptin, 4-hydroxynonenal (4-HNE) were from Calbiochem (La Jolla, CA)

Isolation and culture of human monocyte-derived macrophages

Human mononuclear cells were isolated from buffy coats by density gradient centrifugation, and purified, as described elsewhere [91]. Buffy coats were washed to remove platelets before they were layered on Ficoll. After gradient centrifugation, mononuclear cells were collected and washed to remove Ficoll. Washed mononuclear cells were resuspended in culture medium containing 15% human AB serum and incubated in Teflon bags for 14 days. Subsequently, the monocyto-derived human macrophages were incubated for 2 hours 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. After removal of nonadherent cells by washing,

adherent macrophages were incubated in culture medium containing 5% human AB serum for 48 hours prior to use.

The cell culture medium consisted of RPMI 1640 (Gibco BRL) supplemented with 2 mmol/L L-alanyl-L-glutamine (GLUTAMAX-1), 1% v/v nonessential amino acids, 1 mmol/l sodium pyruvate, 100 U/l Penicillin and 100 µg/ml Streptomycin and 10 mmol/l HEPES (Fluka). All solutions were routinely tested for endotoxin.

Lipoproteins

Human LDL was prepared by ultracentrifugation, using a TL-100 ultracentrifuge (Beckman Instruments) equipped with a TLA-100.4 fixed-angle rotor, as described previously [92]. The LDL fraction was concentrated with a centrifugal filter device (Centricon-100 concentrators from Amicon) and was further purified by gel filtration chromatography on excellulose GF-5 columns (Pierce). LDL was sterilized by filtration through a 0.2 µm pore size syringe filter. LDL was then diluted in phosphate-buffered saline (PBS) to a protein concentration of 3 mg/ml and was oxidized for 24 hours at 37°C with 25 µmol/l $CuSO_4$ as described by Esterbauer et al [93]. The freshly prepared OxLDL was concentrated by ultrafiltration in Centricon-100 concentrators (Amicon), and further purified by size exclusion chromatography on excellulose GF-5 columns (Pierce). Protein concentrations of all LDL samples were determined with bicinchoninic acid (Pierce) using bovine serum albumin as a standard.

Generation of siRNA

Small interfering RNA (siRNA) directed against glutathione reductase, 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 gene obtained from Dr. Dieter Werner (University of Heidelberg, Germany), or human glutaredoxin gene kindly provided by Dr. John J. Mieyal (Case Western

Reserve University). Double-stranded RNA was synthesized with the T7 enzyme mix and 22bp 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, Eugene, OR). Human macrophages were transfected for 6 hours at 37°C with 0.5 μg siRNA. Transfection was performed under serum-free conditions with the lipid-based 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 Negative control in siRNA experiments.

Glutaredoxin activity assay

24 hours after macrophages (150,000/well) were transfected with siRNA, the cells were cultured in RPMI for another 24 hours. The medium was removed. 200 μl lysis buffer (adapted from Ref. 42) (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, 2 μl/ml DMSO) was added to each well and the plates were gently shaken for 10 min at 4°C. The lysates were collected and cleared by spinning. Protein content was measured with the DC Lowry kit from Biorad using BSA as standard.

Glutaredoxin activity was assayed using the standard radiolabel method [74] by our collaborator, Dr. Mieyel. Briefly, an aliquot of BSA-SSG[^{35}S] was added to prewarmed lysate (30°C) with yeast glutathione reductase (GR) (Sigma, St. Louis, MO) (2 units/ml, final). Addition of ice-cold trichloroacetic acid (10%, final) precipitated the assay mixture at 0.5, 1, 2, and 3 min. After centrifugation, the supernatants were analyzed for [^{35}S] by scintillation counting. Glutaredoxin activity is presented as the total rates of *de*glutathionylation which are the slopes of [^{35}S]GSH released *versus* time. The rates were expressed as nanomoles of product/min/mg of cellular protein [42].

Glutathione reductase activity assay

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

Measurement of cellular DNA

The quantity of DNA was determined fluorometrically using the PicoGreen DNA Quantization Kit (Molecular Probes, Eugene, OR). Fluorescence was measured in a FUSION plate reader (Packard) at an excitation wavelength of 485 nm and an emission wavelength of 535 nm. The amount of DNA marks the cell numbers.

Cytotoxicity assay

Cytotoxicity of OxLDL to human macrophages was estimated by measuring leakage of radioactivity from cells pre-loaded with [8-^{3}H]adenine (1 mCi/ml, Amersham), as described by Reid and Mitchinson [94]. Macrophages were loaded with [8-^{3}H]adenine in culture medium with 5% human AB serum for 2 hours and washed twice with PBS for 5 min at 37°C. After cell stimulation, medium of samples were assessed for radioactivity using a liquid scintillation counter. Intracellular radioactivity was determined by lysing the cells with 1% (w/v) Triton X-100 followed by liquid scintillation counting. Leakage of radioactivity into the medium was calculated as a percentage of the total. Total radioactivity per well was always proportional to the cell number, as determined by DNA measurement. Control experiments also confirmed that adenine loading did not interfere with any of the other assays.

Determination of cellular glutathione

Macrophages were rinsed with PBS and scraped off the Aclar. Each sample was divided into three equal aliquots (500 μl). One was used for the determination of DNA as a measure of cell number. One was used for the determination of oxidized glutathione (GSSG) and the last one was used for the determination of total glutathione (GSH+GSSG). Samples were prepared according to Senft et al [95] with modifications. Briefly, the samples designated for GSSG determination were supplemented with N-ethylmaleimide (6 mM) to alkylate free thiol groups. Proteins were precipitated with 100 μl 20% TCA in 20 mM HCl. 250 μl 1 M KPi (pH 7.0) was added to 150 μl clean supernatants to neutralize the pH value. The resulted sample was diluted with 250 μl 0.1 M KPi (pH 7.0) and further reduced with fresh DTT (final concentration 9 mM) for 60 min at room temperature. Glutathione was conjugated with 3.7 mM o-phthalaldehyde and separated by reverse phase HPLC as described by Paroni et al [90]. HPLC separation was performed on a Jasco HPLC system equipped with a spectrofluorometer (FP-920, Jasco Inc.). The excitation and emission wavelength were set at 340 nm and 420 nm, respectively. Glutathione was separated with an isocratic solution (21 mM propionate in 36 mM Na/Pi buffer (pH 6.5)/acetonitrile, 95:5 by vol.) on a Brownlee 22 cm C18 ODS analytical column (5 μm) protected by a Browmlee 3 cm C18 ODS guard column (5 μm). Flow rate was 1.2 ml/min. Levels of reduced glutathione (GSH) were calculated as the difference between GSH+GSSG and GSSG.

Determination of protein-bound glutathione

Macrophages were collected by scraping after being washed with PBS. Each sample was split into two aliquots, one for the determination of DNA (500 μl) and one for the determination of protein-bound glutathione (1000 μl, PSSG). Proteins were precipitated by TCA solution as described before. Protein pellets were dissolved in 150 μl 0.1 M NaOH and diluted with 250 μl 1 M KPi (pH 7.0). Glutathione was released by reduction with 14 mM DTT for 1 hour at room

temperature. Proteins were precipitated by TCA solution, and the supernatants were analyzed for glutathione, as described above.

Statistics

All data points shown are means ± SE of triplicate determinations from 5 independent experiments unless stated otherwise. Data were compared among groups using an unpaired t-test for two groups or one way ANOVA for multiple groups. Results were considered significant at the $P < 0.05$ level. Statistical analysis was performed utilizing SigmaStat (SPSS).

CHAPTER THREE

RESULTS

OxLDL-induced macrophage death and alterations in the thiol redox state

We first examined the effect of OxLDL on the thiol redox state of human macrophages. We found that changes in the thiol redox state occurred prior to macrophage death. The GSH/GSSG ratio is one of the principal determinants of cellular redox state, and alterations in the redox state can lead to cellular dysfunction and cell death [33]. To determine whether OxLDL altered the thiol redox state in macrophages, we measured intracellular GSH_{tot} and GSSG levels in macrophages in the early (24 hours) of OxLDL toxicity. Levels of reduced glutathione (GSH) were expressed as the difference between GSH_{tot} and GSSG. The ratio of GSH/GSSG was calculated using reduced glutathione (GSH) level divided by GSSG level. OxLDL at 10 μg/ml, 25 μg/ml, and 50 μg/ml decreased the GSH/GSSG ratio by 13%, 26% and 40%, respectively. But this decrease in the GSH/GSSG ratio was not accompanied by a statistically significant decrease in macrophage viability (Figure 4A). However, we showed previously that the dose-response curve for OxLDL-induced cell death shifts to the left when incubation time increased to 48 h or 72 h [23], indicating that significant alterations in the celluar redox state precede the onset of macrophage death. After 72 hours of OxLDL treatment, all macrophages had lysed [23].

Both increased accumulation of GSSG and loss of GSH, e.g. due to GSH efflux or GSH conjugation, can contribute to decreases in the GSH/GSSG ratio [96]. While we observed a dose-dependent decrease in GSH, surprisingly we did not observe a significant increase in GSSG level (Figure 4B), implying that in human macrophages the OxLDL-induced decreases in the GSH/GSSG ratio were primarily due to a depletion of GSH rather than an increased accumulation of GSSG.

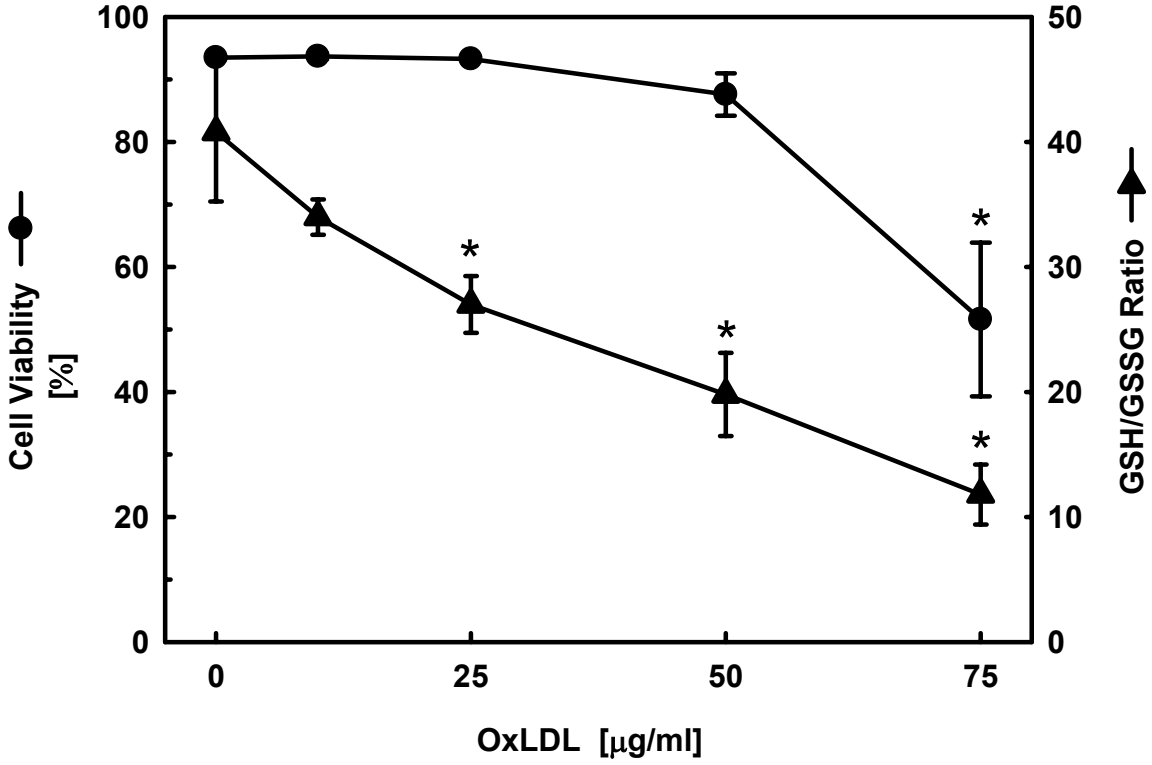

Figure 4A: Effects of OxLDL on cell viability (●) and the GSH/GSSG Ratio (▲). Macrophage viability was assessed with the [^{3}H]adenine release method after stimulation with OxLDL for 24 hours. Cytotoxicity is expressed as the percentage of total cellular radioactivity released into the supernatant. Total cellular radioactivity was measured after cell lysis in the presence of 1% (w/v) Triton X-100 with scintillation counting. Determinations of GSH_{tot} and GSSG were performed as described in "Material and Methods". Levels of reduced glutathione (GSH) were calculated as the difference between GSH_{tot} and GSSG. Results are expressed as mean ± SE. *P < 0.05 vs control without OxLDL treatment.

Next, we examined whether the decrease in intracellular GSH results from GSH efflux. Cells may release glutathione into the extracellular space. Reduced GSH is the major transport form which plays a significant role in GSH turnover [97]. The release of GSSG has also been observed under conditions of oxidative stress in different cell types including erythrocytes [98] and eye lens [99]. As

predicted, the extracellular GSH_{tot} level was increased when cells were treated with 10 μg/ml and 25 μg/ml OxLDL. However, after OxLDL concentration was

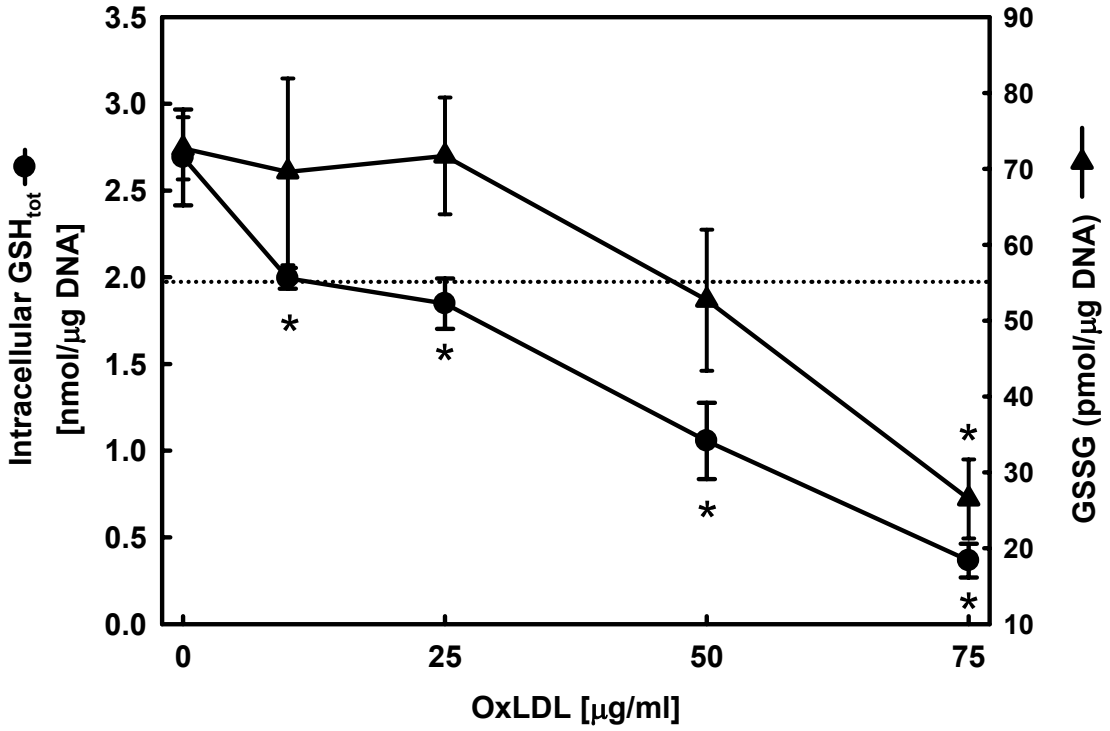

Figure 4B: Effects of OxLDL on intracellular GSH_{tot} (●) and the GSSG level (▲). Intracellular GSH_{tot} and the GSSG level were determined as described in "Material and Methods". Results are expressed as mean ± SE. *P < 0.05 vs control without OxLDL treatment. The dotted line represents the concentration of GSH determined by HPLC in the culture medium.

increased to 50 μg/ml, GSH_{tot} exhibited similar level to that at 0 μg/ml OxLDL treatment, indicating that GSH efflux had stopped at that point. Nevertheless, when the concentration of OxLDL was increased to 75 μg/ml, the decrease in intracellular GSH level may be due to cell leakage. Therefore, our data suggest that GSH efflux does not appear to contribute to OxLDL-induced decreases in the GSH/GSSG ratio.

Another explanation for the decrease in GSH would be GSH conjugation with aldehydes. Several reports suggest that glutathione can conjugate with

aldehydes and be exported from the cell [71, 100, 102]. OxLDL has been shown to cause an initial decrease of GSH in the human monocyte cell line THP-1 and macrophages isolated from C3H/HEJ strain of mice [25]. This fall in glutathione occurs as a result of its oxidation or conjugation with aldehydes and exportation from the cell [25]. To examine whether 4-HNE has an effect similar to OxLDL,

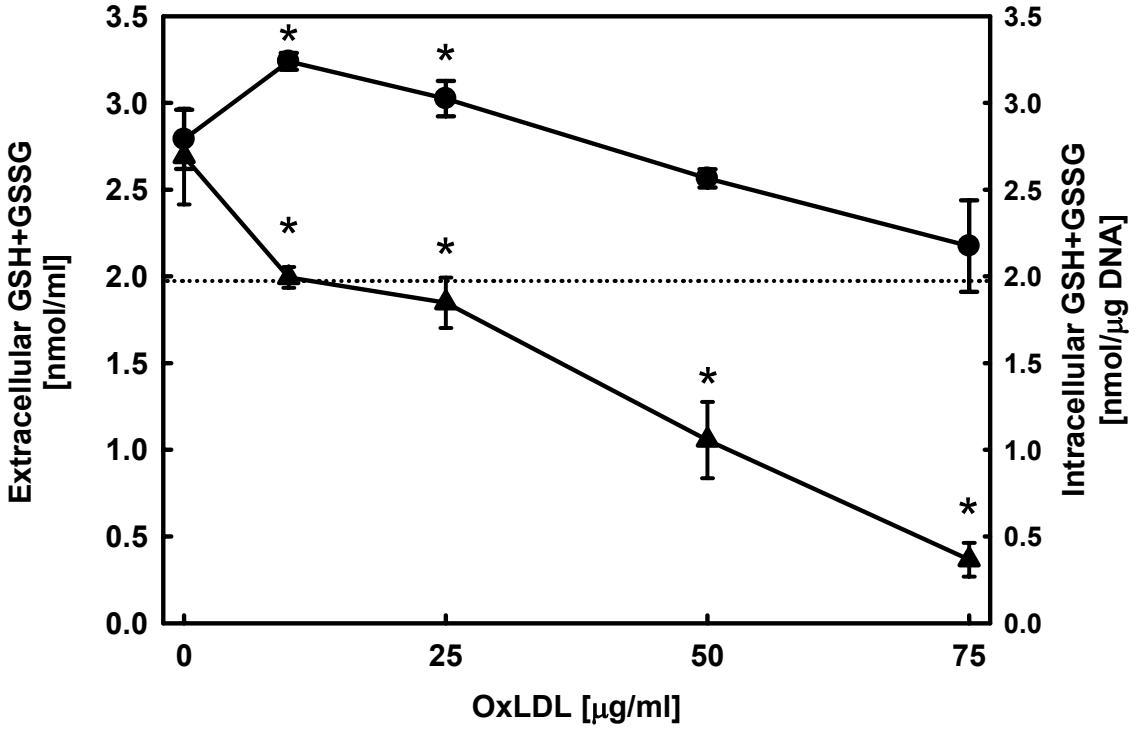

Figure 5: Effects of OxLDL on intra- and extra-cellular levels of glutathione. [^{3}H]Adenine-loaded human monocyte-derived macrophages were incubated for 24 hours with OxLDL at the indicated concentrations. Intracellular GSH+GSSG (▲) were measured as described in "Material and Methods". The culture medium was used to determine extracellular GSH + GSSG (●). Results are expressed as mean ± SE. *P < 0.05 vs control without OxLDL treatment.

we measured cell viability, the GSH/GSSG ratio, and GSH_{tot} level in macrophages treated with increasing dose of 4-HNE for 24 hours . We found that 4-HNE-induced macrophage death appeared to be preceded by and correlate to a dose-dependent decrease in the GSH/GSSG ratio (Figure 6A). As expected, both GSH_{tot} levels and GSSG level decreased (Figure 6B). However, the decrease in the GSH/GSSG ratio indicates that GSSG levels

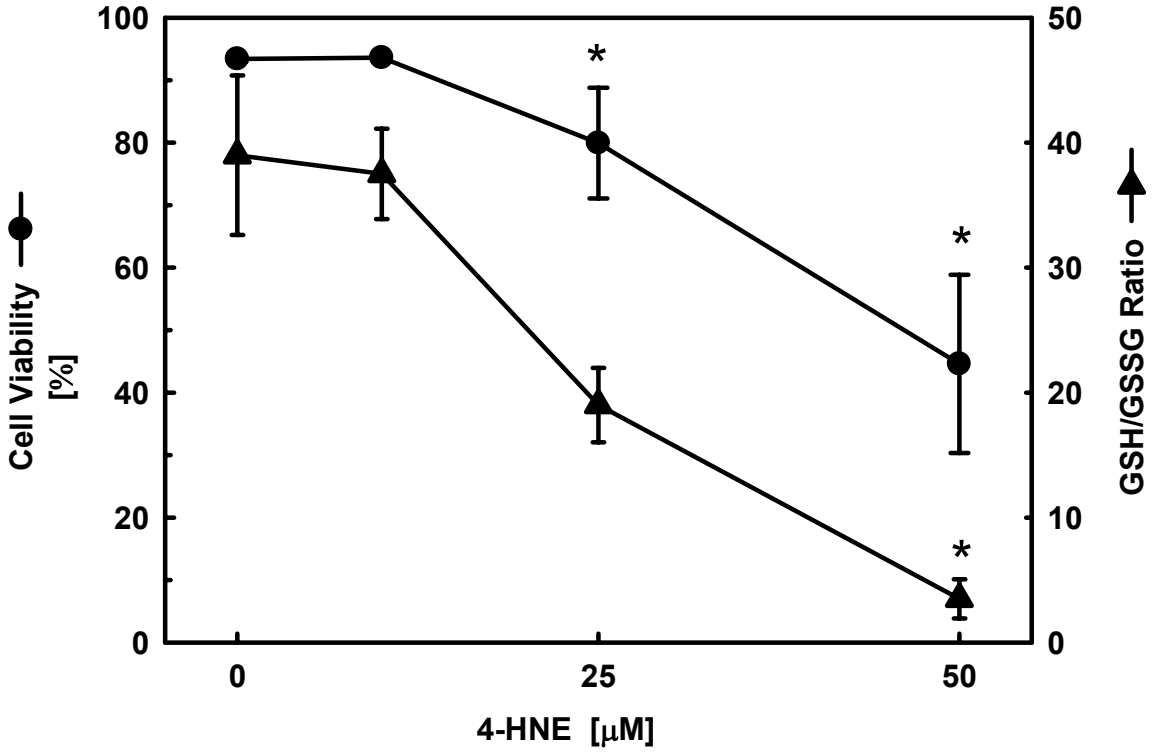

Figure 6A: Effects of 4-HNE on cell viability and the GSH/GSSG Ratio. [^{3}H]Adenine-loaded human monocyte-derived macrophages were incubated for 24 hours with 4-hydroxynoneal (4-HNE) at the indicated concentrations. Cell viability (●) and the GSH/GSSG ratio (▲) were determined as described in "Material and Methods". Results are expressed as mean ± SE. *P < 0.05 vs control without 4-HNE treatment. n=4

decreased proportionally slower than GSH. The slower decrease in GSSG may result from suppressed activity of glutathione reductase (GR) by 4-HNE. Added

4-HNE decreased the GSH level and GSH/GSSG ratio at the onset of macrophage death, similar to the effects observed with OxLDL treatment, i.e. a two-fold decrease in GSH, and a two-fold decrease in the GSH/GSSG ratio (Figure 6A). The data suggested that 4-HNE or other aldehydes present in OxLDL may account for the depletion of GSH observed during OxLDL-induced macrophage death.

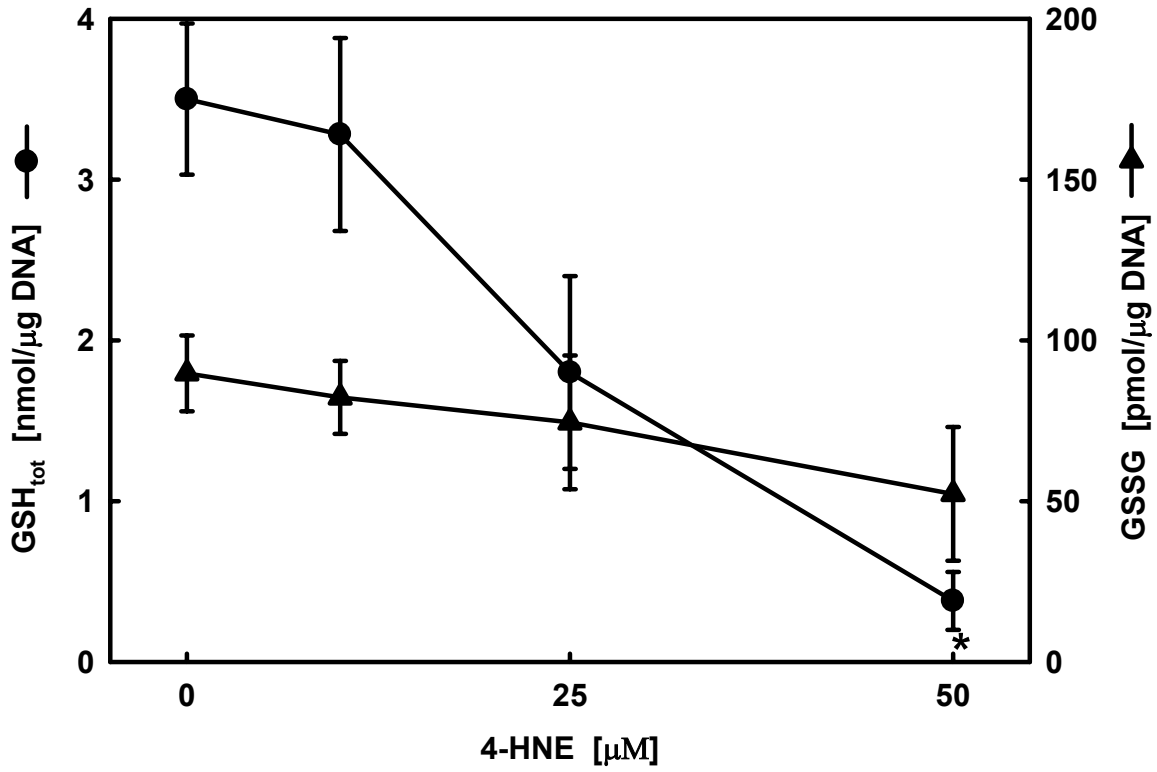

Figure 6B: Effects of OxLDL on intracellular GSH_{tot} (●) and the GSSG level (▲). Intracellular GSH_{tot} and the GSSG level were determined as described in "Material and Methods". Results are expressed as mean ± SE. *P < 0.05 vs control without 4-HNE treatment. n=4

Role of GSH depletion in OxLDL-induced macrophage death

Having demonstrated that the depletion of GSH contributes to OxLDL-induced macrophage death, we studied whether the depletion of GSH was

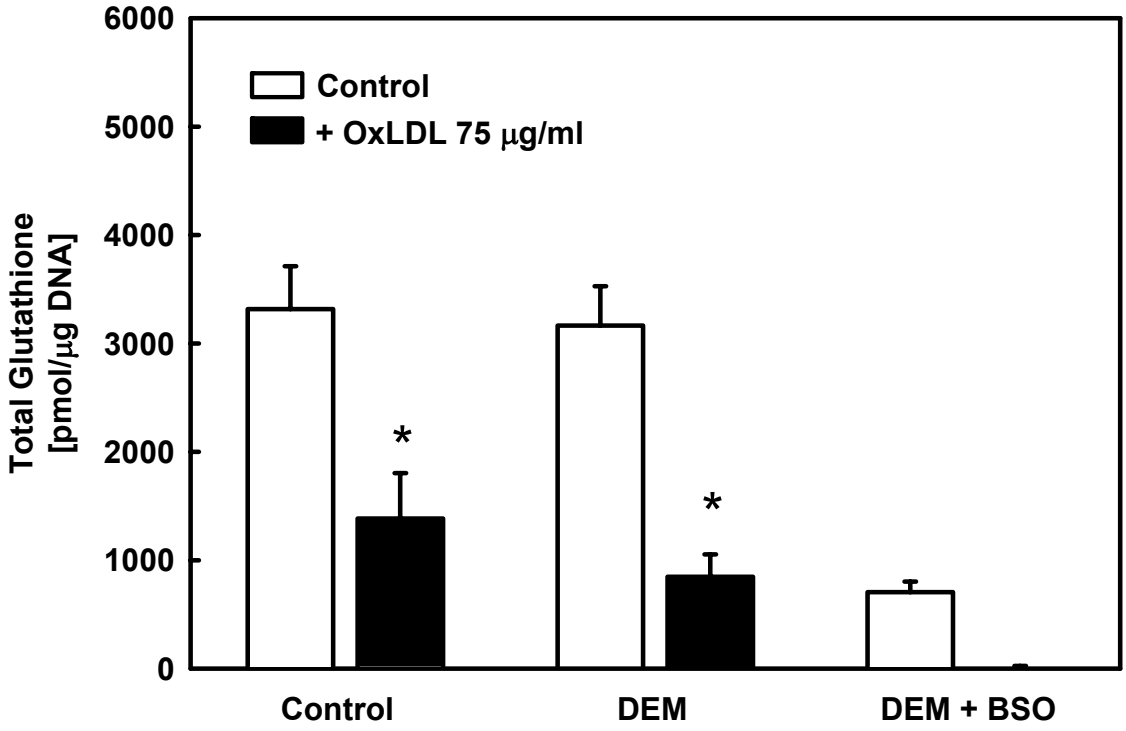

Figure 7A: Effect of DEM, BSO and OxLDL on glutathione levels in macrophages. [^{3}H]Adenine-loaded macrophages were preincubated for 2 h in either medium alone or the thiol alkylating agent diethyl maleate (DEM, 1 mM). Cell were then treated for 24 hours with either medium only (□) or with OxLDL (75 μg/ml) (■). The GSH synthesis inhibitor buthionine sulfoximine (BSO, 0.3 mM) was present where indicated. GSH_{tot} was determined as described in "Material and Methods". Results are expressed as mean ± SE. *P < 0.05 vs control without OxLDL treatment. n=3

sufficient to cause macrophage death. Buthionine sulphoximine (BSO) is an inhibitor of γ-glutamyl-cysteinesynthetase (γ-GCS) and has been used in a

number of biochemical and pharmacological studies as a specific agent for inhibiting GSH biosynthesis and thereby induce GSH depletion [102]. Another

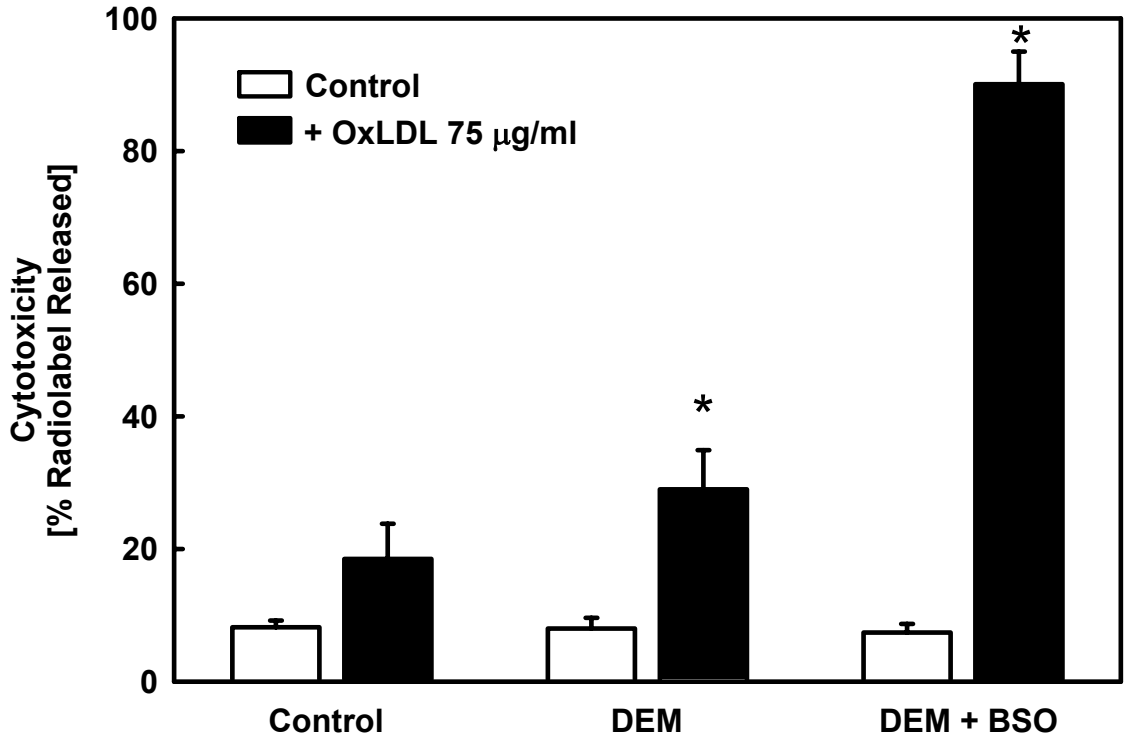

Figure 7B: Effect of glutathione depletion on OxLDL-induced macrophage injury. To reduce intracellular glutathione levels, [^{3}H]Adenine-loaded macrophages were preincubated for 2 hours in either medium alone or the thiol alkylating agent diethyl maleate (DEM, 1 mM). Cell were then treated for 24 hours with either medium only (□) or with OxLDL (75 μg/ml) (■). The GSH synthesis inhibitor buthionine sulfoximine (BSO, 0.3 mM) was present where indicated. Macrophage viability was measured as described in "Material and Methods". Results are expressed as mean ± SE. *P < 0.05 vs control without OxLDL treatment. n=3

widely used drug diethyl maleamide (DEM) depletes GSH through its conjugation with GSH [103, 104]. Kuzuya and coworkers reported that a decrease in the GSH levels induced by the pre-treatment of cells with BSO enhanced the cytotoxicity of OxLDL in vascular endothelial cells [105]. To determine the role of GSH depletion in macrophage injury, we utilized BSO and DEM to reduce intracellular GSH level. Macrophages were preincubated for 2 hours in either medium alone or 1 mM DEM prior to be treated for 24 hours with either medium only or with OxLDL (75 μg/ml) in presence or absence of BSO (0.3 mM). Macrophages intracellular GSH level (Figure 7A) and viability were then measured (Figure 7B). DEM, and DEM plus BSO in the absence of OxLDL depleted GSH_{tot} by 23.1% and 92.4%, but cell viability was not significantly decreased. This result indicates that despite low GSH_{tot} level, cytotoxicity is not elevated confirming that GSH depletion alone is not sufficient to promote macrophage death in the absence of oxidative stress. Addition of OxLDL dramatically increased cell cytotoxicity in cells incubated with DEM+BSO compared with other treatments, suggesting that the depletion of GSH potentiated macrophage injury induced by OxLDL. Furthermore, DEM and DEM+BSO depleted GSH_{tot} and GSSG level, resulting in an increase in the GSH/GSSG ratio from 27 to 33 and 45, respectively (data not shown). The increase in the GSH/GSSG ratio indicates that GSSG levels decreased proportionally faster than GSH. The possible explanation is that glutathione reductase (GR) is more effective without OxLDL toxicity, thereby converting more GSSG to reduced GSH.

To further investigate whether the decreased GSH/GSSG ratio was independent of the formation of peroxyl radical, we loaded cells with 250 μM Trolox which is a peroxyl radical scavenger to inhibit the formation of peroxyl radical. Previously, our lab demonstrated that addition of OxLDL accelerated the rate of intracellular ROS formation by 3.6 fold and this increase was inhibited by 93% in the presence of the peroxyl radical scavenger Trolox [23]. Here we show that Trolox restored cell viability in OxLDL-treated macrophages, but restored neither GSH_{tot} level nor the GSH/GSSG ratio (Figure 8), indicating that the

decreases in the GSH_{tot} and the GSG/GSSG ratio were independent or upstream of the peroxyl radical formation.

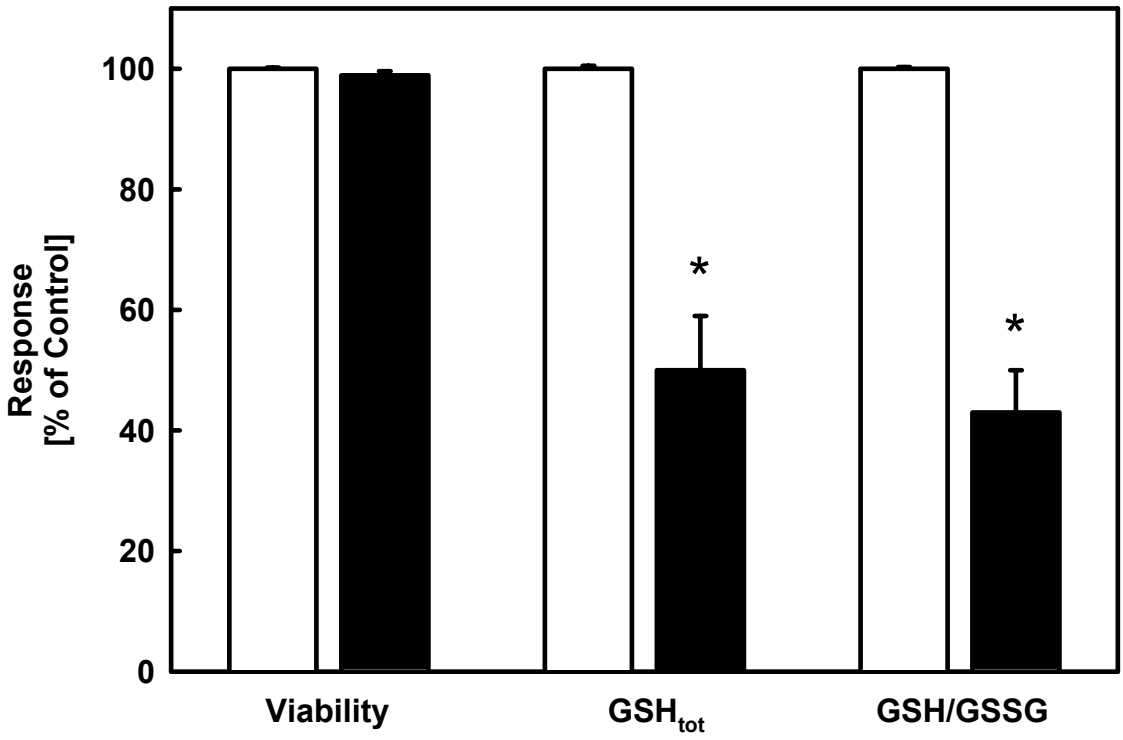

Figure 8: Effect of Trolox on OxLDL-induced alterations in cell viability, GSH_{tot} Levels and the GSH/GSSG ratio. [^{3}H]Adenine-loaded macrophages were treated for 24 hours with either medium only (□) or with OxLDL (75 µg/ml) plus Trolox (250 µM) (■). Cell viability, GSH_{tot} and the GSH/GSSG ratio were determined as described in “Material and Methods”. Results are expressed as mean ± SE. *P < 0.05 vs control without Trolox treatment.

Protein-*S*-glutathionylation may mediate OxLDL-induced macrophage death

Both increased ROS formation and changes in the thiol redox potential, i.e. accumulation of GSSG or decreased GSH/GSSG ratio, can induce protein-*S*-glutathionylation [85]. We therefore investigated whether protein-*S*-glutathionylation may involve in OxLDL toxicity. To this end, macrophages were exposed to OxLDL (100 μg/ml) for 24 hours. Cell cytotoxicity and PSSG levels were measured. OxLDL alone killed macrophages (Figure 9A), but did not significantly increase PSSG (Figure 9B). However, it is possible that PSSG have either leaked out of the macrophages or the increase in PSSG was limited to a few selected proteins and overall made make up <<10% of macrophage proteins. To promote the accumulation of PSSG and enhance the detectability of PSSG formed, we preincubated macrophages for 1 hour with 0.2 mM 1,3-bis[2-chloroethyl]-1-nitrosourea (BCNU), a glutathione reductase (GR) inhibitor, which causes the accumulation of GSSG and therefore limites the ability of Grx to deglutathionylate PSSG [99]. Subsequently, macrophages were exposed to OxLDL (100 μg/ml) for 24 h. Interestingly, BCNU alone had no effect on cell cytotoxicity or PSSG, but potentiated both OxLDL-induced PSSG formation (Figure 9A) and cytotoxicity (Figure 9B), demonstrating that PSSG accumulation is not detectable unless GR is blocked by BCNU. However, inhibition of GR by BCNU may cause sufficient GSSG accumulation to promote non-enzymatic, formation of PSSG. However, that is unlikely as this reaction generally only occurs at the GSH/GSSG ratios < 10 [77].

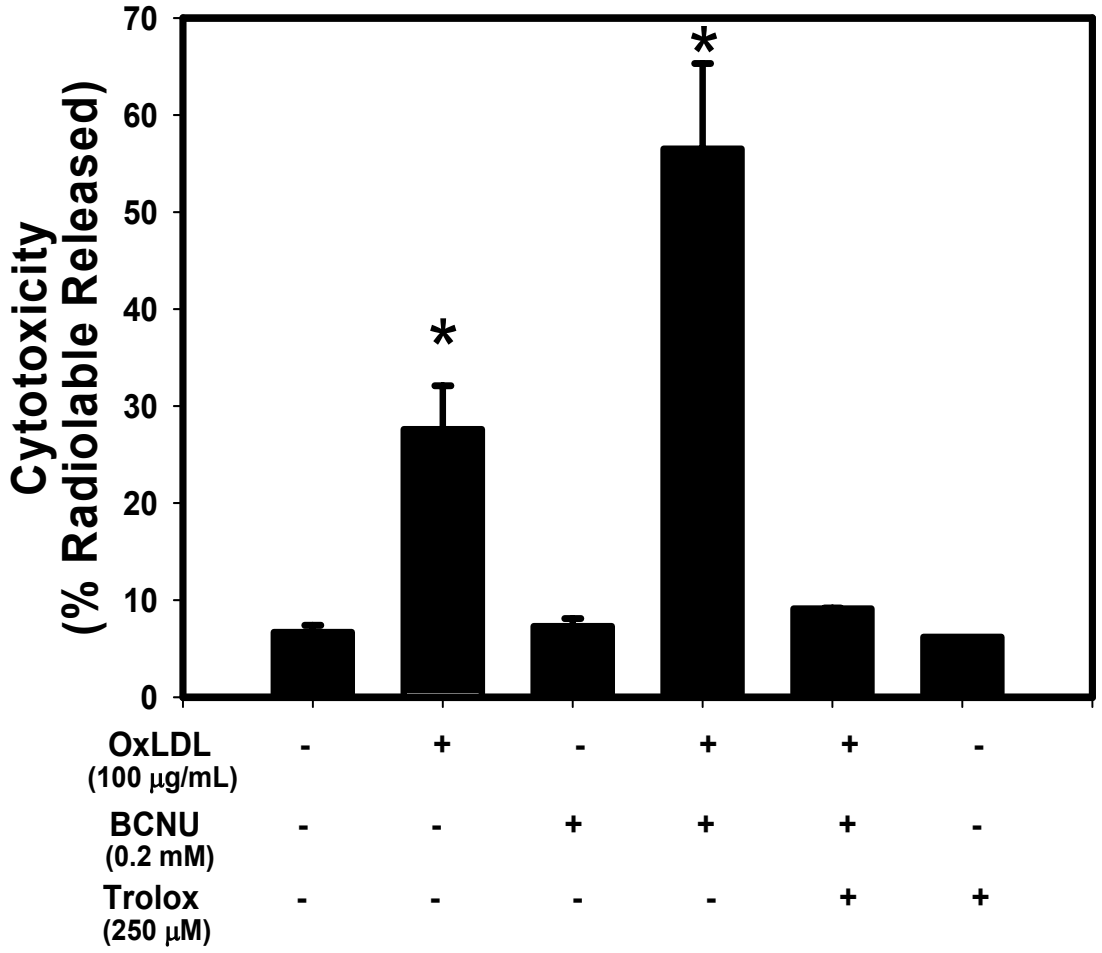

Figure 9A: Effect of BCNU, and Trolox on macrophage death. Human macrophages were incubated either in the absence or presence of OxLDL (100 μg/ml) or OxLDL+BCNU (0.2 mM). Trolox (250 μM) was present where indicated. Macrophage cytotoxicity was measured as described in "Material and Methods". Results are expressed as mean ± SE. *P < 0.05 vs control without OxLDL, BCNU and Trolox treatments. n=3

Alternatively, enhanced ROS formation may oxidize PSH into PSOH, which readily reacts with GSH to form PSSG. Under conditions of limited deglutathionylation, i.e. with BCNU present, this would lead to the accumulation of PSSG. To determine whether OxLDL-induced ROS contributes to PSSG

formation in OxLDL-induced macrophages, macrophages were exposed to OxLDL (100 μg/ml) for 24 hours in the absence or presence of the potent peroxyl

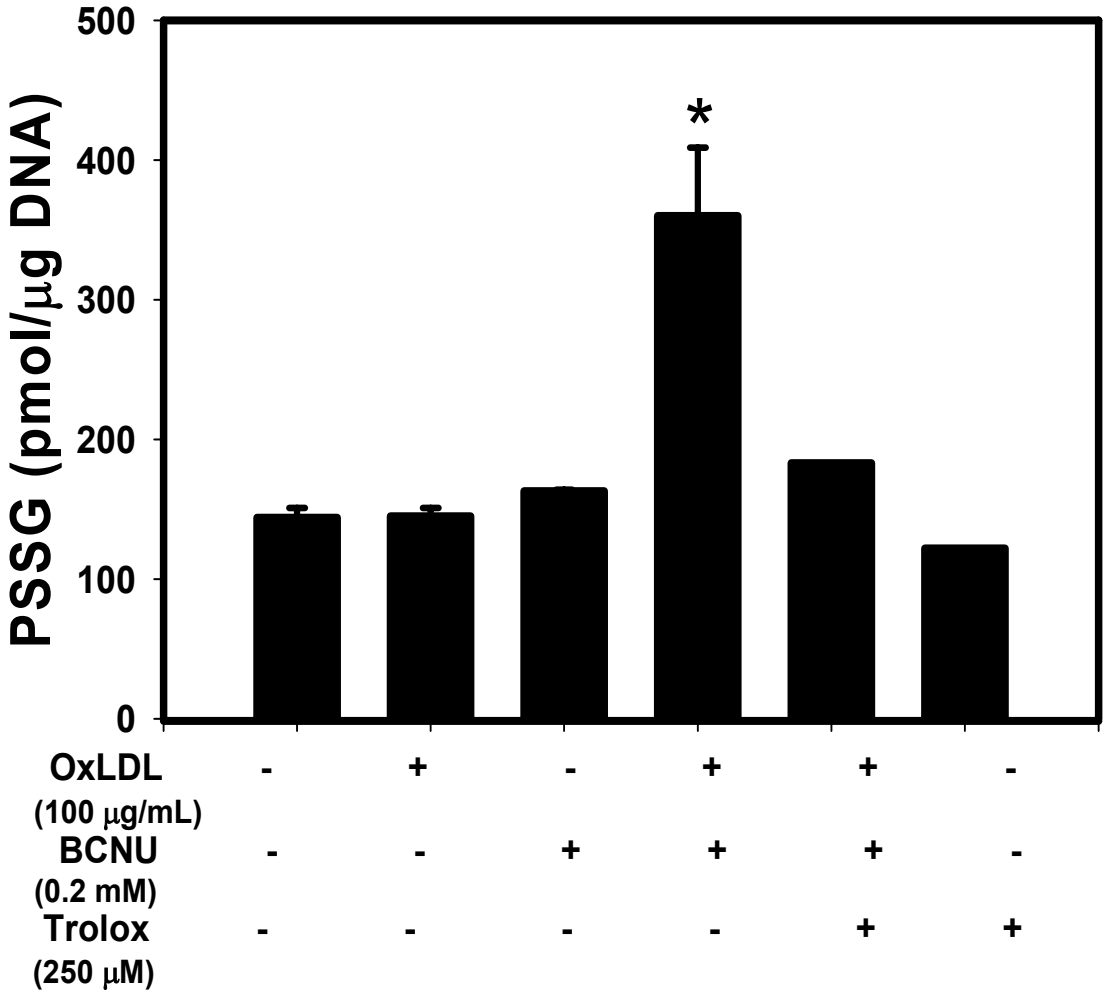

Figure 9B: Effect of BCNU, and Trolox on macrophage P-SSG level. Human macrophages were incubated either in the absence or presence of OxLDL (100 μg/ml) or OxLDL+BCNU (0.2 mM). Trolox (250 μM) was present where indicated. PSSG were analyzed as described in "Material and Methods". Results are expressed as mean ± SE. *P < 0.05 vs control without OxLDL, BCNU and Trolox treatments. n=2

radical scavenger Trolox. Interestingly, Trolox blocked PSSG formation and OxLDL-induced macrophage death. This result suggests that OxLDL–induced macrophage death may involve peroxyl radical mediated formation of PSOH and the subsequent generation of PSSG.

A role for the glutaredoxin (GRx) in protecting macrophages from OxLDL-induced cell injury

OxLDL-induced protein-*S*-glutathionylation (Figures 9A and B) may contribute to macrophage injury. We therefore examined if GRx, which is the primary enzyme involved in the *de*glutathionylation of PSSG [42], is required to

Table 1: Effect of siRNA directed against glutathione reductase and glutaredoxin on GRx activity and GR activity. siRNA directed against glutathione reductase and glutaredoxin were prepared with the Dicer Kit (Gene Therapy Systems, Eugene, OR) and transfected into human macrophages. Lysates were prepared and assayed for protein content and GRx or GR activity as described under "Materials and Methods". The activities are presented as nanomoles/min/mg. Results are expressed as mean ± SD. n=1

	GR Activity nmol/min/mg	**GRx Activity nmol/min/ml**
Non-transfection	**42.5±3.9**	**2.2±0.03**
Mock	**40.9±2.5**	**2.3±1.4**
Anti-FITC-GL2	**40.8±0.5**	**2.4±0.4**
Anti-GRx	**40.9±0.9**	**0.6±1.7**
Anti-GR	**38.0±1.7**	**2.2±3.1**

protect macrophages against OxLDL toxicity. Macrophages were transfected with siRNA directed against GRx as evidenced by a 70% decrease in GRx activity (Table 1). We observed no difference in GRx activity in mock, anti-GR and anti-FITC-GL2 transfected cells compared to non-transfected macrophages, suggesting that siRNA against GRx specifically and efficiently blocked the

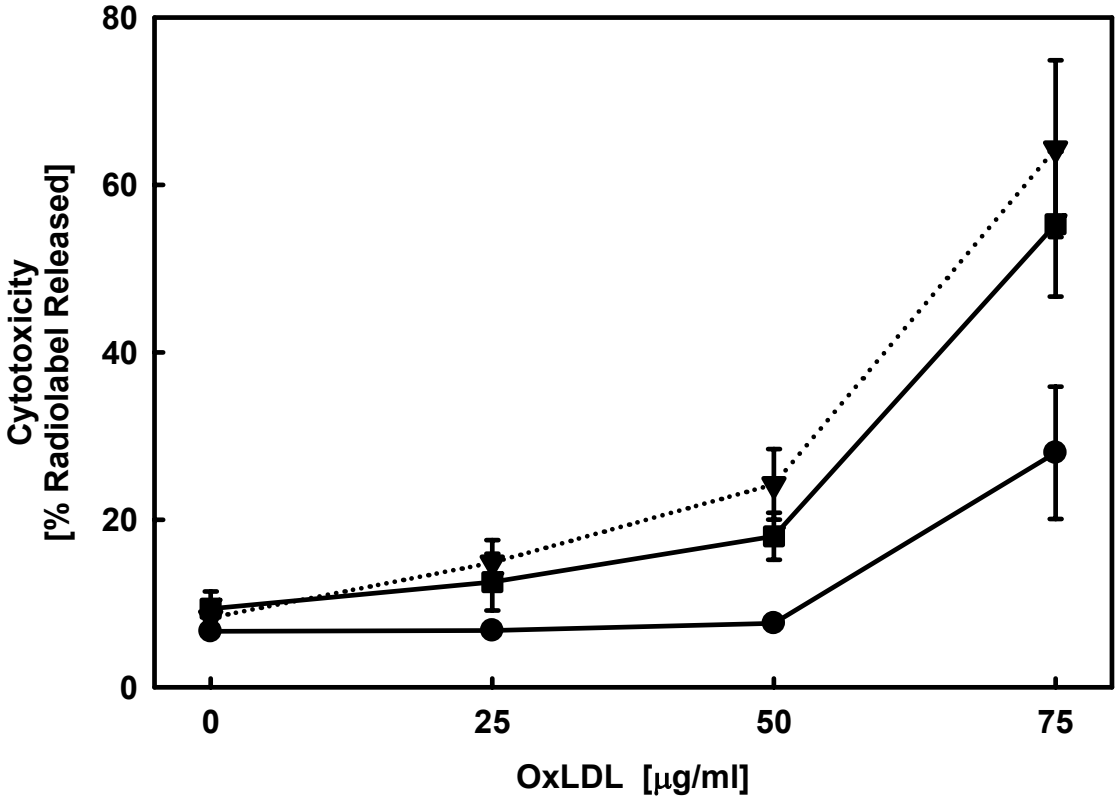

Figure 10: Effect of siRNA directed against glutathione reductase and glutaredoxin on OxLDL-induced cell death. siRNA directed against glutathione reductase and glutaredoxin were prepared with the Dicer Kit (Gene Therapy Systems, Eugene, OR) and transfected into human macrophages. Cells were loaded with [^{3}H]adenine for 2 hours and washed. Mock-transfected (●) and macrophages transfected with siRNA directed against either GR (■) or Grx (▲) were incubated for 24 h with OxLDL at the indicated concentrations and cell viability was measured as described in “Materials and Methords”. Results are expressed as mean ± SE. n=4

expression of GRx. Importantly, when macrophages were transfected with siRNA against GRx or GR, the dose-dependent curves for OxLDL-induced macrophage death was shifted to the left (Figure 10]. Transfection of human macrophages with siRNA directed against luciferase GL2 showed no effect on OxLDL-induced cytotoxicity (data not shown). This result suggests that GRx protects macrophages from OxLDL toxicity.

We also attempted to quantitate the expression level of GRx and GR in siRNA transfected macrophages with RNA_{ase} Protection Assay with no success due to the insufficient sensitivity of this method for human macrophage cells.

A role for the glutathione reductase (GR) in protecting macrophages from OxLDL-induced cell injury

Glutathione reductase (GR) is the enzyme responsible for maintaining the GSH/GSSG ratio by converting intracellular GSSG into GSH. GRx catalyzes the *de*glutathionylation of PSSG. GRx is recycled to the reduced enzyme by GSH, resulting in the formation of GSSG, which in turn is regenerated to GSH by GR [42]. Thus, GR plays important role in GRx turnover. We would expect that the inhibition of GR activity not only enhances GSSG accumulation and PSSG formation but also prevents the *de*glutathionylation of PSSG. Hence, inhibiting the GR would sensitize macrophages to OxLDL-induced cell injury. To test this hypothesis, we examined the dose dependency of OxLDL-induced macrophage death at the absence or presence of the GR inhibitor, BCNU. In the presence of BCNU, the LD_{50} of OxLDL decreased 2.5-fold, demonstrating that BCNU potentiated OxLDL-induced macrophage death (Figure 11).

To further examine if glutathione reductase (GR) is critical in protecting macrophages from OxLDL-induced injury. Macrophages were transfected with siRNA against human GR. We observed a minor (10%) but significant decrease in GR activity with antiGR siRNA transfection (Table 1). There is no significant difference in GR activity in mock, anti-GR and anti-FITC-GL2 transfected cells

compared to non-transfected macrophages, suggesting that siRNA against GR specifically and efficiently blocked the expression of GR. As expected, the does-dependent curve for OxLDL-induced macrophage death shifted to left for human macrophages transfected with siRNA directed against GR (Figure 10), suggesting that GR also plays a protective role in OxLDL-induced macrophage death.

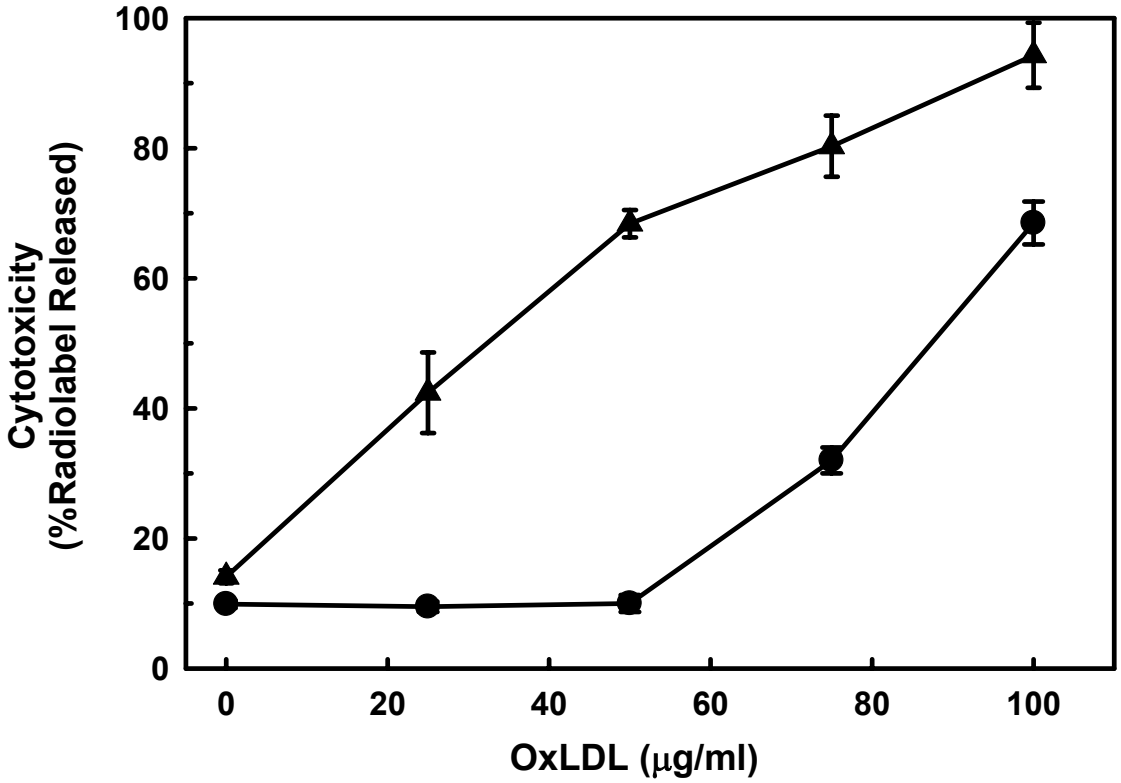

Figure 11: Effect of the glutathione reductase inhibitor BCNU on OxLDL-induced macrophage death. [^{3}H] Adenine-loaded macrophages were incubated for 18 hours with OxLDL at the indicated concentrations. Then vehicle (●) or BCNU (▲) was added to a final concentration of 0.2 mM. Cytotoxicity was measured after 48 hours. Results are expressed as mean ± SE. n=4

CHAPTER FOUR
DISCUSSION

Macrophage death is believed to be an important event in the pathogenesis of human atherosclerosis and can be induced by OxLDL *in vitro* [10, 20]. The mechanisms underlying OxLDL-induced macrophages death is unclear. In the present study, we provide evidence that OxLDL-induced cell injury in human macrophage is mediated by two independent signals: decreased GSH/GSSG ratio and peroxyl radical formation. Both signals are required for cell death. Furthermore, we provide evidence that macrophage death induced by OxLDL involves the formation of PSSG, and that the GRx/GR enzyme system protects macrophage from OxLDL-induced cell injury.

Previously, we demonstrated that OxLDL-induced oxidative stress promotes cell lysis in human macrophages, a process that involves the formation of peroxides, but not superoxide [23]. Scavengers of peroxyl radicals such as Trolox prevent macrophage lysis, implicating peroxyl radicals in macrophage lysis induced by OxLDL [23]. However, peroxyl radical formation alone may not be sufficient to induce macrophage death because increasing the OxLDL concentration from 80 μg/ml to 150 μg/ml showed no further increase in the rate of peroxyl radical formation, yet the rate of macrophage death tripled [23]. One explanation could therefore be that a second signal or component of OxLDL is involved in OxLDL-induced macrophage damage as we discuss below.

In this research, we show that a change of the glutathione redox state in human macrophages contributes to OxLDL-induced macrophage death. 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 [33]. Hence, the additional signal required for OxLDL toxicity in addition to peroxyl radical formation may involve changes of the GSH/GSSG ratio. Indeed, we found that OxLDL-induced macrophage death inversely correlated to the GSH/GSSG ratio. After 24 hours of OxLDL treatment, a dose-dependent decrease in the cellular GSH/GSSG ratio preceded

macrophage death, indicating that significant alteration in the cellular redox state may lead to macrophage death.

However, changes in cellular redox state alone, i.e. depletion of GSH or decreased GSH/GSSG ratio, cannot explain OxLDL-induced macrophage death. This conclusion is based on our observation that depletion of intracellular glutathione with the alkylating agent diethyl maleamide (NEM) and glutathione synthesis inhibitor *L*-buthionine-sulfoximine (BSO) alone did not promote cell death, but potentiated OxLDL-induced macrophage injury. This result is in agreement with GSH depletion studies in thymocytes, which demonstrate that treatment with BSO depletes GSH by 90% but is non-toxic [107, 108]. Interestingly, NEM and BSO+NEM increased the GSH/GSSG ratio accompanying with the depletion of GSH in human macrophages (data is not shown). Those results suggested that the GSH depletion in the absence of oxidative stress can not lead to the decrease in the GSH/GSSG ratio and fails to cause cell death, implying that the decreased GSH/GSSG ratio is required in OxLDL-induced cell death. Furthermore, our experiments with Trolox, a peroxyl radical scavenger, showed that Trolox blocked OxLDL-induced cell death, but restored neither intracellular glutathione levels nor the GSH/GSSG ratio, indicating that glutathione depletion and a decreased GSH/GSSG ratio induced by OxLDL occur independent or upstream of peroxyl radical formation. However, partial restoration of intracellular GSH stores with glutathione diethyl ester did not improve the GSH/GSSG ratio, but substantially reduced OxLDL toxicity (data not shown). Together, our data suggest that both a decrease in the GSH/GSSG ratio and peroxyl radical formation are required for OxLDL-induced macrophage death, and those two signals are independent. However, inhibition of peroxyl radical formation by Trolox completely restore cell viability, implying that the depletion of GSH and the decreased GSH/GSSG ratio are the fundamental defect that renders macrophages susceptible to cell injury by the peroxyl radical formation, but alone is not sufficient to kill macrophages.

Two mechanisms can lead to a decreased GSH/GSSG ratio: accumulation of GSSG or loss of GSH with insufficient conversion of GSSG into

GSH [96]. Under conditions of marked oxidative stress, intracellular GSSG can increase substantially [102]. Multiple mechanisms can contribute to this increase: i) non-enzymatic oxidation of GSH ii) GSH is converted to GSSG by glutathione peroxidase (GPx), which catalyzes the reduction of H_2O_2 and other peroxides. iii) conversion of PSSG to PSH by GRx-SH and subsequent recycling of Grx-SSG to Grx-SH (Figure 3). We observed an apparent decrease in GSSG level in the macrophages treated with OxLDL, implying that the collapse of the GSH/GSSG ratio in response to OxLDL might be precipitated primarily by a decrease in GSH, not the accumulation of GSSG. However, the experiments performed in DEM+BSO treated macrophages demonstrated that macrophages will maintain the GSH/GSSG ratio even under GSH depleted conditions as one would expect if GR remains fully functional. Close examination of the effect of OxLDL on GSH and GSSG levels reveals that in fact GSSG levels do not decrease at the same rate as GSH. This could either be due to loss of GR activity and/or enhanced GSSG formation. Further experiments are needed to determine which mechanism leads to the decreased ratio.

Although the molecular mechanism involved is not clear, GSH depletion is likely to occur via GSH efflux or GSH conjugates including GSSG and 4-HNE-GS. If glutathione is transported out of the cell as GSH and GSSG, we would have detected a significant increase in extracellular GSH_{tot} that correlated with the decrease in intracellular GSH level. However, that was not observed. Therefore, GSH efflux does not appear to be responsible for the depletion of GSH. OxLDL contains high concentration of aldehydes including 4-HNE [21, 105]. Darley-Usmar and coworkers demonstrated that pure 4-HNE and OxLDL deplete GSH from monocyte-like THP-1 cells [25]. Hartley and coworkers suggested that the mechanism by which 4-HNE depletes the GSH involves the conjugation of GSH with 4-HNE to form 4-HNE-GS which is transported out of cells [109]. If GSH conjugates with alkenals, alkylated conjugates would probably not be reduced by DTT [109] because DTT only reduces reversible sulfhydryl modifications such as intramolecular disulfide, mixed disulfide, or sulfenate [77]. Thus, the method described by Paroni et al. would not detect the change in extracellular GSH_{tot}.

Given the ability of GSH conjugates to lead to the depletion of GSH in OxLDL-treated human macrophages, we reasoned that the 4-HNE may mimic the cytotoxicity of OxLDL in human macrophages. As predicted, 4-HNE caused macrophage death which was correlated with the depletion of GSH and a decrease in the GSH/GSSG ratio. However, Trolox did not protect against 4-HNE-induced toxicity (data not shown), suggesting that HNE cannot be the agent or alkenal in OxLDL that does the killing. Together, these data indicate that the pathway of decreased GSH/GSSG ratio contributes to OxLDL-induced macrophage death. Furthermore, the decrease in the GSH/GSSG ratio is not caused by the reduction of cellular glutathione alone. Therefore, our data suggest that GSH depletion appears to contribute to OxLDL-induced cell injury and may result from the conjugation between GSH and OxLDL-derived alkenals.

The shift in the ratio of GSH/GSSG changes the cellular redox state. Changes in the cellular redox environment can alter signal transduction, DNA and RNA synthesis, protein synthesis, enzyme activation, and even regulation of the cell cycle [34-36]. Thus, a significant decrease in the GSH/GSSG ratio may also lead to cell death. Changes in the intracellular redox state induced by a decreasing GSH/GSSG ratio or the increased formation of peroxyl radicals can promote the formation of mixed disulfides between protein cysteine residues and GSH, a process referred to as protein-*S*-glutathionylation [42, 77]. Protein-*S*-glutathionylation alters (mostly inhibits) protein function and/or activity [85]. Protein-*S*-glutathionylation is reversible, however, once it reaches a critical level, macrophages are likely to become dysfunctional and die. We therefore speculate that the accumulation of PSSG may contribute to OxLDL-induced macrophage death. However, we could not detect a significant increase in PSSG in response to OxLDL (100 μg/ml), unless we added BCNU. One possible explanation is that only a small number of proteins critical for cell survival are *S*-glutathionylated. Unless these proteins would make up at least 10% of the total macrophage protein, we would not have detected an increase in total PSSG. Alternatively, cytosolic PSSG may have leaked out of the dying cells. However, in the presence of BCNU, OxLDL stimulated PSSG formation, which correlated also

with a dramatic increase in cell cytotoxicity. Inhibition of glutathione reductase (GR) with BCNU promotes the accumulation of GSSG. This may slow down the regeneration of Grx-SSG and thus decrease the rate of PSSG regeneration, effectively resulting in the accumulation of PSSG. These data show that OxLDL promotes protein-*S*-glutathionylation and that increased PSSG formation appears to play a critical role in OxLDL toxicity in human macrophages. We also observed that Trolox blocked PSSG formation and restored cell viability. This result suggests that peroxyl radical formation appears to be involved in the accumulation of PSSG.

The mechanisms by which PSSG accumulated in human macrophages treated with OxLDL are unclear. The formation of PSSG by GSH transfer from GSSG to PSH would require that the intracellular GSH/GSSG ratio have to drop below 10-13 [110]. But in most cells and tissues, such a low GSH/GSSG ratio in the cytosol is rare because of the ability of many cells to export GSSG, a protective response against oxidative stress [111]. Indeed, when OxLDL at 75 ug/ml killed half of macrophages, the GSH/GSSG ratio was still well above 10. Hence, high GSSG levels or a decrease in the GSH/GSSG ratio is not a likely mechanism for PSSG formation in OxLDL-induced macrophages. Alternatively, ROS, in particular radicals, could promote PSSG [42, 64, 77]. Indeed, treatment of cells with Trolox inhibited the formation of PSSG and blocked OxLDL-induced macrophage death, suggesting that peroxyl radicals may contribute to PSSG formation. It is also possible that the depletion of GSH promotes the formation of PSSG in OxLDL-induced macrophages. In support of this mechanism, Glutaredoxin (GRX) is the primary enzyme involved in the *de*glutathionylation of PSSG requiring GSH as cofactor [42, 77]. The depletion of GSH would therefore reduce the level of active GRx and hence prevent the *de*glutathionylation of PSSG, resulting in the accumulation of PSSG in OxLDL-induced macrophages. To test this hypothesis, human macrophages could be supplemented with GSH prior to treatment with OxLDL. GSH is taken up only in small amounts by cells [112]. But GSH diethyl ester transports more efficiently into cells where it is rapidly hydrolyzed to GSH monoethyl and then to GSH [113]. Hence, GSH

diethyl ester can be added in culture medium to increase GSH level. We would expect that the formation of PSSG and cell cytotoxicity induced by OxLDL will be decreased after the addition of GSH diethyl ester.

Accumulation of PSSG has been reported in different cell types under a variety of oxidative conditions [49]. GRx has been characterized in vitro as a specific catalyst for the reduction of PSSG [42]. When the activity of GRx was downregulated 70% by siRNA interference, cell toxicity induced by OxLDL was increased 30%, indicating that the involvement of PSSG formation in OxLDL-induced macrophage death and an important role for GRx in protecting macrophages from OxLDL toxicity.

GR can catalyze the reduction of GSSG, and *in vivo*, GR is also required for the conversion of GRx into its reduced, i.e. active state [73]. Inhibiting GR expression with siRNA against GR dramatically increased OxLDL-induced macrophage death, demonstrating that inhibition of GR activity would increase both GSSG accumulation and PSSG formation, and prevent the *de*glutathionylation of PSSG due to a reduction in the level of active GRx. Furthermore, treatment with GR inhibitor BCNU, which causes the accumulation of GSSG, sensitized macrophages to OxLDL toxicity, indicating the importance of thiol oxidation and the protective role of GR in OxLDL-induced macrophage death. However, it is not likely that OxLDL directly inactivates GR. This conclusion is based on our observation that OxLDL alone did not promote a detectable increase in PSSG level. But when GR activity was inhibited by BCNU, the PSSG level induced by OxLDL dramatically increased.

Our data are summarized in the hypothetical model of OxLDL-induced human macrophage death shown in figure 12. Our model suggests that OxLDL contains lipid hydroperoxides (LOOH) which are most likely the precursors of peroxyl radicals [24]. The formation of peroxides can be blocked by peroxyl radical scavenger Trolox and GPx, which reduce LOOH to LOH using GSH as substrate. But the detoxification of peroxides via GPx produces more GSSG [114, 115]. The study of Asmis et al. [23] suggests that the formation of peroxyl radical causes mitochondrial dysfucntion, thereby promoting macrophage lysis. Here we

show that alterations in the thiol redox state contribute to OxLDL-induced macrophage death. Our data demonstrate that the depletion of GSH in macrophages results from the conjugation of GSH with 4-HNE or other alkenals presented in OxLDL particle rather than GSH efflux. However, the depletion of GSH alone cannot explain the decrease in the GSH/GSSG ratio because under normal physiological condition, GR converts GSSG to GSH faster than GSSG is formed, resulting in the reduced GSSG level. Hence, macrophage death is only observed under conditions where GSSG levels decreases to a lesser extent than GSH, i.e. due to either loss of GR activity and/or enhanced

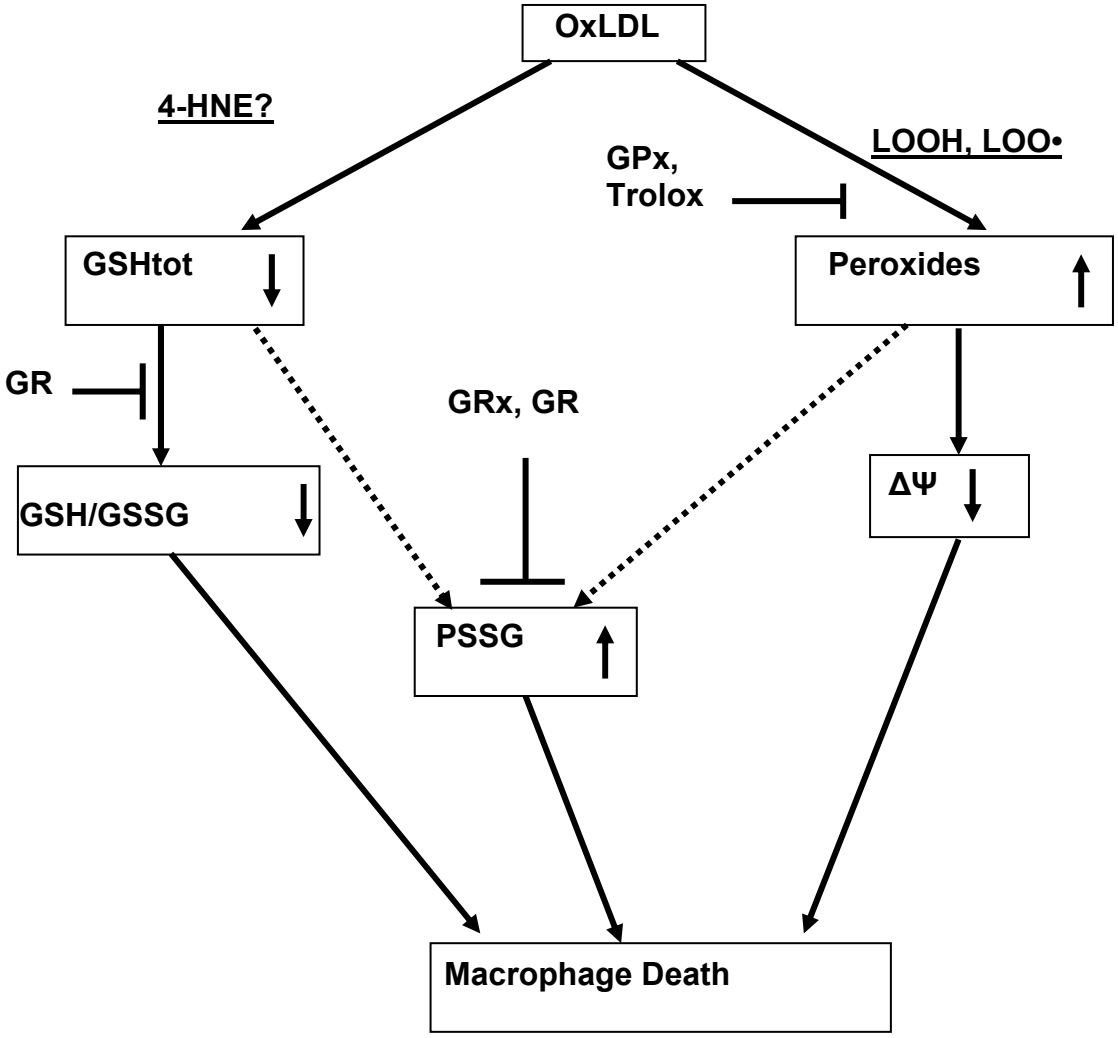

Figure 12: Hypothetical Model of OxLDL-induced macrophage death

GSSG formation, a decreased GSH/GSSG ratio can be observed. It is conceivable that increased GR activity can inhibit the decreased GSH/GSSG ratio as a result of reducing the accumulation of GSSG.

The change in the thiol redox state and peroxyl radical formation is independent pathways in OxLDL toxicity and both of them are required for OxLDL-induced macrophage death. The depletion of glutathione or the decreased GSH/GSSG ratio is the fundamental defect that renders macrophages vulnerable to killing by the peroxyl radical formation, but alone is not sufficient to induce macrophage death. Furthermore, Trolox inhibits the formation of PSSG and cell death as result of suppression of peroxide formation. Hence, the formation of peroxides and/or the depletion of GSH may induce the accumulation of PSSG. When the increased formation of PSSG reaches a critical level, macrophages are likely to become dysfunctional and die.

GRx, a catalyst of the *de*glutathionylation of PSSG, and GR required for the regeneration of GSH from GSSG and the restoration of GRx activity, play protective roles in OxLDL-induced macrophage death. Therefore, Grx enzyme system is likely to delay the progression of atherosclerotic lesions through reducing the death of macrophage death. The GRx and GR are important future targets to develop clinical therapies for atherosclerosis associated with oxidative stress.

APPENDIX

ABBREVIATIONS:

BCNU: 1,3-bis[2-Chloroethyl]-1-nitrosourea
BSO: *L*-buthionine-sulfoximine
DEM: Diethyl maleate
DTT: Dithiothreitol
γ-GCS: γ-glutamylcysteine synthetase
GPx: Glutathione peroxidase
GR: Glutathione reductase
GRx: Glutaredoxin
GS: Glutathione synthetase
GSH: Reduced glutathione or γ-glu-cys-gly
GST: Glutathione-s-transferase
GSSG: Glutathione disulfide
4-HNE: 4-Hydroxynonenal
IKK: IkB kinase
LOOH: Lipid hydroperoxides
NEM: N-ethylmaleimide
OxLDL: Oxidatively modified LDL
PSSG: Protein-glutathionyl-mixed disulfides
ROS: Reactive oxygen species
TRx: Thioredoxin
TRxR: Thioredoxin reductase
TDOR: Thiol-disulfide oxidoreductase
Trolox: 6-Hydroxy-2,5,7,8-tetremethyl-chroman-2-carboxylic acid

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