Induction of Glutathione Synthesis in Macrophages by Oxidized Low-Density Lipoproteins Is Mediated by Consensus Antioxidant Response Elements

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Abstract—The uptake of oxidized low-density lipoproteins (oxLDL) by macrophages leading to conversion into foam cells is a seminal event in atherogenesis. Excessive accumulation of oxLDL can cause oxidative stress in foam cells leading to cell death and the progression and destabilization of atherosclerotic lesions. Oxidative stress induces a protective compensatory increase in the synthesis of the endogenous antioxidant glutathione (GSH). Glutamate-cysteine ligase (GCL) is the rate-limiting enzyme in GSH synthesis and is composed of a catalytic subunit (GCLC) and a modifier subunit (GCLM), which are products of separate genes. Treatment of RAW 264.7 mouse macrophages and mouse peritoneal macrophages with oxLDL (30 µg/mL) induces increased expression of both Gclc and Gclm in vitro. The increase in mRNA occurs in part via increased transcription as demonstrated with luciferase reporter constructs. The promoters for both GCLC and GCLM contain consensus antioxidant response elements (AREs). Electrophoretic mobility shift assays revealed induction of nuclear factor binding to these AREs after treatment of RAW 264.7 cells and mouse peritoneal macrophages with oxLDL. Nuclear factor binding to the AREs is diminished by a single base pair substitution in the core sequence. Site-directed mutagenesis of the AREs within the Gclc and Gclm promoters resulted in a decrease of oxLDL-induced luciferase activity. Supershift analyses revealed that oxLDL stimulates binding of the transcription factors Nrf1, Nrf2, and c-jun to the AREs. These data suggest that AREs play a direct role in mediating the induction of GSH synthesis by oxLDL and in protecting macrophages against oxidized lipid-induced oxidative stress. (Circ Res. 2003;92:386-393.)

Key Words: arteriosclerosis ■ oxidized low-density lipoproteins ■ glutathione ■ macrophages ■ oxidative stress

The uptake of oxidized low-density lipoproteins (oxLDL) by macrophages and their conversion into foam cells is recognized as a seminal event in the development of atherosclerosis.1 The oxidation of LDL is thought to occur within the vascular wall and lead to a variety of proatherogenic responses such as activation of the endothelium and recruitment of inflammatory cells, impaired endothelium-dependent vascular relaxation, migration and proliferation of smooth muscle cells, promotion of procoagulant properties of vascular cells, and alteration of signaling pathways.2–4 Multiple cell types within the arterial wall contribute to the formation of oxLDL, although the exact mechanisms are not known. However, evidence suggests that mediators such as metal ions, lipoxygenases, cyclooxygenases, reactive nitrogen species, and products of myeloperoxidase may play a role.4,5 Foam cell formation and turnover play important roles in both the initiation and the progression and ultimate instability of advanced lesions. Excessive accumulation of lipids derived from oxLDL induces oxidative stress in foam cells and causes a compensatory increase in the synthesis of the endogenous antioxidant glutathione (GSH).6–10 GSH is an abundant intracellular thiol that serves to buffer changes in the cellular redox state and provides protection from reactive electrophiles or free radicals. Clinical studies have demonstrated a relationship between low serum GSH levels and coronary artery disease.11 In addition, apo E−/− mice with increased GSH levels exhibit reduced cell-mediated oxidation of LDL and reduced atherosclerotic lesion development.12 This protective effect of serum GSH is thought to occur by inhibiting extracellular LDL oxidation, whereas the intracellular GSH content of macrophages determines the response to the cytotoxic effects of oxidized lipids and other pro-oxidants.10,13 Furthermore, inhibition of GSH synthesis potentiates oxLDL toxicity in macrophages.10 This is of particular significance because the death of macrophages is a prominent feature of atherosclerotic lesions and likely contributes to plaque rupture.14

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The rate-limiting enzyme in GSH synthesis is glutamate-cysteine ligase (GCL), a heterodimeric enzyme composed of a catalytic subunit GCLC and a modifier subunit GCLM, which are encoded by separate genes.\textsuperscript{15,16} The promoters of both GCL subunit genes contain consensus antioxidant response elements (AREs)\textsuperscript{17–19} that play essential roles in regulating the cellular responses to oxidative stress\textsuperscript{20} and mediate induction of both GCL subunits in several different cell types.\textsuperscript{17,21–23} The increase in GSH synthesis after treatment with oxLDL occurs via an increase in GCL activity and protein and increases in the expression of both subunits of GCL.\textsuperscript{6,9–24} However, the molecular mechanisms for this induction remain poorly understood. Thus, the present study (72 C57BL/6J mice 4 days after intraperitoneal injection of thioglycolate purchased from Ambion.)

**Chemicals and Reagents**

LDL (density 1.019 to 1.063 g/mL) was prepared from the plasma of fasted, healthy male donors by sequential density gradient ultracentrifugation. This protocol involving human volunteers was approved by the University of Washington Human Subjects Review Committee. Native LDL (natLDL) was dialyzed overnight in PBS, filtered, and diluted to 300 μg protein/mL in PBS containing 25 μmol/L BHT. OxLDL was prepared by diluting to 300 μg/mL natLDL in PBS without BHT and followed by incubation with 5 μmol/L CuSO\textsubscript{4} for 24 hours at 37°C. The oxidation reaction was stopped with addition of BHT (25 μmol/L final concentration), but the BHT in both the oxLDL and natLDL was removed by dialysis before addition to the cells and the lipoproteins were stored under nitrogen. The final preparations of oxLDL contained between 40 to 50 μmol/mg protein of thiobarbituric reactive substances (TBARS), as a measure of the extent of oxidation. Lipofectamine reagent was purchased from Gibco BRL (Gibco-BRL) and Trizol reagent was purchased from Ambion.

**Tissue Culture**

RAW 264.7 cells, a murine macrophage cell line (ATCC, Manassas, Va), were grown in DMEM (Gibco BRL) containing 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin at 37°C in a 95% O\textsubscript{2}/5% CO\textsubscript{2} humidified environment. Mouse peritoneal macrophages (10^6 cells per animal on average) were obtained through peritoneal lavage with ice-cold PBS from C57BL/6J mice 4 days after intraperitoneal injection of thioglycollate (72 μg/mouse). The C57BL/6J mice were purchased from the Jackson Laboratories (Bar Harbor, Maine) and used according to a protocol approved by the University of Washington Institutional Animal Care and Use Committee. Cells were purified by adhesion to tissue culture dishes for 24 hours after harvesting prior to the experiments.

**Real-Time Quantitative PCR**

RAW 264.7 cells were treated with 30 μg/mL natLDL or oxLDL in DMEM plus 10% FBS, and total RNA was harvested from the cells at various time points using Trizol reagent, according to the manufacturer’s protocol. Gclc and Gclm mRNA message levels were obtained by quantitative real-time PCR of cDNA generated from 2 μg total RNA for each sample. Quantitative real-time PCR used a fluorogenic 5’ nuclease assay with primers and probes directed against the 3’-most intron/exon boundary of both the Gclc and Gclm genes. The sequences of the primers and probes are as follows (all sequences 5’→3’): Gclc sense primer, ATGGTGACACCTGGCAGTTATT; Gclc antisense primer, TGTCTTTGTTGATGTACGAGTTTT; Gclm probe, CATTAGTCTCCAGATGCTCTCTCTTATAA; Gclm sense primer, GCCACGATTGTAGCTGCTT;

**Reporter Constructs and Plasmids**

The Gclc reporter constructs were prepared as follows: a 7-kb EcoRI/BamHI fragment containing exon 1 and –5.6-kb of Gclm promoter was initially subcloned into pBluescript (Stratagene). A 6.5-kb XhoI/NcoI fragment containing 6447 bp of Gclm promoter was then shuttled into pGL3 Basic to yield the full-length 6.5-kb Gclc luciferase reporter construct. Deletion constructs were generated by digesting the 6.5-kb Gclc reporter construct with SacI or KpnI followed by recircularization of the plasmid to yield reporter constructs containing 3869 bp (3.8-kb Gclm) and 1281 bp (1.3-kb Gclm) of Gclm promoter, respectively. Site-directed mutagenesis of the ARE sites in the Gclm promoter was carried out by Bio S&T (Bio S&T) and confirmed by DNA sequencing.

To prepare the Gclm luciferase reporter constructs, a 1292-bp NcoI fragment of the mouse Gclm promoter\textsuperscript{25} was inserted into the NcoI site of the luciferase reporter vector pGL3 (Promega). Reporter constructs containing mutations within the ARE (m1 and m2) were engineered by separately amplifying a –1685-bp F/MutR fragment and a MutF/GL2R fragment, overlapping fragments that contained the mutation of interest within the region of overlap. The full-length fragment was amplified by addition of –1685-bp F and GL2R primers to the reaction, and the product was blunt-end ligated into a shuttle vector. The 1292-bp NcoI/NcoI fragment containing the mutation was then excised and ligated into the NcoI site of the pGL3 vector. All amplifications were performed using Vent polymerase (New England Biolabs). The sequences of the mutagenesis primers were (5’→3’):

ARE m1F, GAGGTTCCTGCATGTTCTGCTCAGAGAAA-

CAGCTCC
ARE m1R, GGAGCTTTTCTGGAAGACAAAGGACTAAGCAGA-
AACCTC
ARE m2F, TTTCTCGGAGGTTTCAAATAATGCTATCTC-
TTCAAG
ARE m2R, CCGGAAAGACAAATAATGAGCTAATTTGAAACC-
TCACCCGAGAAA

**Reporter Assays**

RAW 264.7 cells were seeded into 35-mm plates 24 hours before transfection at 3 × 10^4 cells/well. Cells were transfected using Lipofectamine reagent according to the manufacturer’s protocol. Briefly, cells were transfected with 1.5 μg DNA/well for 5 hours. All samples were cotransfected with a CMV- Renilla luciferase construct (Promega) to compensate for variances in transfection efficiencies. Twenty-four hours after beginning transfection the medium was changed to complete medium or medium containing 30 μg/mL natLDL or oxLDL. Cells were treated for 24 hours at which time they were harvested to obtain cell extracts for luciferase assays. Cells were harvested using passive lysis buffer (Promega) according to the manufacturer’s protocol. Luciferase assays were performed using the Dual Luciferase Reporter System (Promega) according to the manufacturer’s protocol and samples were measured in duplicate on a Lumat model 9507 luminometer (EG&G Berthold).

**Statistical Analysis**

All data are expressed as mean±SEM. Significant differences between means were determined by using the student’s two-tailed t test. All experiments were performed at least three times and representative results are shown.

**Results**

**OxLDL Increases Gclc and Gclm mRNA Levels**

To determine whether oxLDL induces an increase in Gcl subunit message levels, mouse RAW 264.7 cells were treated with 30 μg/mL natLDL or oxLDL in the tissue culture medium for up to 24 hours (Figure 1). We did not observe any toxicity using this concentration of LDL. Cellular RNA was harvested for real-time quantitative PCR determination of Gclc and Gclm mRNA levels. Note that RNA was collected from untreated control cells at each time point as Gclc and Gclm subunit message levels fluctuate with changing cellular confluence. We observed induction of both Gclc and Gclm steady-state mRNA levels in response to treatment with oxLDL with induction occurring as early as 3 hours and, for the modifier subunit, persisting for 24 hours (Figure 1). Peak induction for both subunit mRNAs occurred after 6 hours of treatment. In contrast to oxLDL, treatment with natLDL does not appreciably alter message levels for either subunit. Induction of Gclm message after oxLDL treatment is consistently more robust than that observed for Gclc, with a peak 16-fold induction of Gclm message versus 8-fold induction for Gclc message relative to untreated controls.

**OxLDL Induces Expression of Gclc and Gclm Reporter Constructs**

An increase in the steady-state mRNA levels could be due to both increased de novo transcription and/or message stability. To determine whether the observed increases in Gclc and Gclm mRNA levels after oxLDL treatment are due to transcriptional upregulation, we transfected RAW 264.7 cells with luciferase reporter constructs containing 6.5 kb of the mouse Gclc or 1.3 kb of the mouse Gclm proximal promoter regions. Transiently transfected RAW 264.7 cells were treated with 30 μg/mL natLDL or oxLDL for 24 hours at which time cell extracts were harvested and assayed for luciferase activity. This time point demonstrated maximum luciferase activity as evaluated through time course experiments. OxLDL increased luciferase reporter expression from both the Gclc (Figure 2A) and Gclm (Figure 2B) promoters with a 1.8-fold increase in luciferase activity for the Gclc construct and a 2.5-fold increase observed for the Gclm construct. The activity of the internal standard, Renilla luciferase, was not affected by treatment with oxLDL (data not shown). These data provide evidence for increased transcription of the Gcl subunits as part of a coordinated response to upregulate GSH production upon exposure to oxLDL in macrophages.

As oxLDL triggers an oxidative stress response, we postulated that AREs present within the Gcl subunit promoters mediate the observed increase in transcription after oxLDL treatment. The GCLC promoter contains several putative AREs, of which two distal AREs have been characterized as functional for the human GCLC promoter in response to β-naphthoflavone.\(^ {17} \) Transfection of reporter constructs containing truncated fragments of the mouse Gclc promoter into RAW 264.7 cells and subsequent treatment with oxLDL or natLDL reveal that promoter elements necessary for induction by oxLDL lie between −3.8 kb and −6.5 kb relative to the translation start codon (Figure 2A). This region of mouse Gclc promoter contains two putative AREs bearing semblance to the human ARE3 and ARE4, which mediate induction of human GCLC in response to oxidative stress; hence, we refer to these putative AREs as...
OxLDL Induces Binding of Nuclear Factors to Gclc and Gclm AREs

We conducted electrophoretic mobility shift assays (EMSAs) to determine whether oxLDL induces binding of nuclear factors to AREs located in the mouse Gclc and Gclm subunit promoters. RAW 264.7 cells were treated with oxLDL or natLDL for up to 12 hours during which time cells were harvested at various time points for the preparation of nuclear extracts. Nuclear proteins (5 to 10 μg) were incubated at room temperature for 20 minutes with a radiolabeled double-stranded oligonucleotide corresponding to mouse Gclc ARE3, Gclc ARE4, or Gclm ARE, and the resulting protein:DNA complexes were separated by polyacrylamide gel electrophoresis. As demonstrated in Figure 3, oxLDL induced binding of nuclear factors to both the Gclc ARE3 and ARE4 (Figure 3A) as well as the Gclm ARE (Figure 3B). Addition of excess unlabeled oligonucleotide to the binding reaction successfully competed away nuclear factors, indicative of binding specificity. The time course of induction is similar for all AREs examined, with increased nuclear factor binding occurring within 1 hour after treatment and returning to basal levels by 12 hours. However, for the two Gclc AREs, we observe different patterns of nuclear factor binding, particularly in the magnitude of the changes. The induction is much greater at ARE4 compared with ARE3, whereas the basal level of nuclear factor binding is greater at ARE3. Interestingly, natLDL also strongly induces binding of nuclear factors to these AREs, but the observed induction lags behind the induction after oxLDL treatment. This is perhaps due to oxidation of natLDL by the macrophages in the tissue culture dish. Gclm ARE also exhibits constitutive binding of nuclear factors, but induction is clear by 1 hour of treatment with oxLDL. In contrast to the catalytic subunit AREs, natLDL only slightly induced binding of nuclear factors to the modifier subunit ARE and, similar to the Gclc AREs, the binding is delayed (Figure 3B).

We also conducted gel shift experiments using oligonucleotides for each of three AREs, which contained single base pair mutations within the ARE consensus sequence, and observed that mutations in the AREs strongly inhibited binding of nuclear factors. As a representative example, the reduced binding of nuclear proteins to the mutated ARE4 is presented in Figure 4, lane 7. Furthermore, addition of anti-Nrf1, anti-Nrf2, or anti-c-jun antibodies to the binding reaction resulted in displacement of the protein:DNA binding complex indicating involvement of the Nrf1, Nrf2, and c-jun transcription factors at this site. In contrast, addition of an anti-c-fos antibody had only minor effects. These transcription factors are known to bind to the AREs and influence transcription of certain ARE-containing genes. The displacement by Nrf1, Nrf2, and c-jun could be observed for ARE4 as demonstrated in Figure 4, lanes 3 through 6, and similar results were also observed for ARE3 and Gclm ARE (data not shown).

Mutations in Gclc and Gclm Promoter AREs Attenuate Induction by OxLDL

We transfected RAW 264.7 cells with reporter constructs containing targeted mutations within the AREs of the Gclc and Gclm promoters to assess the functional significance of the AREs in induction of these genes in response to oxLDL. Cells were transiently transfected with a luciferase reporter construct containing 6.5 kb of the Gclc promoter with mutations in either ARE3 or ARE4 or both, and luciferase activities were measured in cell extracts obtained after 24 hours of treatment with oxLDL. The introduced mutation targets the core TGAC sequence of these AREs and contains...
a T>G single base pair transversion. This single base pair mutation in ARE3 diminished constitutive expression of the luciferase reporter but had no effect on induction by oxLDL (Figure 5A). In contrast, the same mutation in ARE4 affected both constitutive and oxLDL-induced expression of the luciferase reporter. Mutation of both AREs resulted in a luciferase expression pattern similar to that observed for the ARE4 mutation alone, suggesting that both ARE3 and ARE4 coordinate to influence constitutive expression, whereas ARE4 predominates in the induction response to oxLDL.

For the Gclm reporter construct, we separately introduced two mutations into the ARE of the luciferase reporter construct containing 1.3 kb of the mouse Gclm promoter. The mut1 mutation is the same mutation introduced into the AREs of the Gclc promoter as described above, and the second mutation (mut2) targets the GCA box just downstream and contains a GCA/TTT triple base pair mutation. These mutated ARE constructs were transiently transfected into RAW 264.7 cells and their ability to drive oxLDL-inducible expression was compared with the wild-type ARE luciferase construct. Figure 5B reveals that both mutations in the Gclm ARE inhibit induction of luciferase expression on oxLDL treatment, with no effect on basal luciferase expression. These data, coupled with the EMSA results, support a functional role for the AREs in the observed induction of Gclc and Gclm expression by oxLDL.

Induction of Gcl mRNA and Binding of Nuclear Proteins to the AREs Is Also Observed in Mouse Peritoneal Macrophages

The experiments described above were all conducted in RAW 264.7 cells, an immortalized macrophage cell line. To test whether oxLDL also induces expression of the Gcl subunits and binding of nuclear proteins to the AREs in primary cells, we conducted real-time quantitative PCR and gel shift experiments with mouse peritoneal macrophages. As indicated in
Figure 6A, oxLDL but not natLDL induced expression of Gclc and Gclm after 6 hours of treatment. Furthermore, oxLDL induced the binding of nuclear proteins to all AREs. As already shown for the RAW 264.7 cells, the transcription factors Nrf1, Nrf2, and c-jun were identified in the primary macrophages. As a representative example, ARE4 is shown in Figure 6B. However, functional analyses of the AREs could not be determined, because peritoneal macrophages could only be transfected with the constructs used previously for the RAW 264.7 cells at very low transfection efficiency (data not shown).

Discussion

Macrophage cell death in response to oxidative stress likely plays a fundamental role in the progression and destabilization of atherosclerotic lesions. Clinical and experimental studies suggest that GSH is a critical component of the endogenous defense mechanisms in macrophages against oxidative stress. Darley-Usmar et al first demonstrated that treatment of THP-1 cells or mouse peritoneal macrophages with oxLDL increases cellular GSH levels. This increase in GSH correlates with increased levels of GCL protein and activity. However, it is not known whether the observed increases in GCL protein and activity reflect posttranslational modifications of the GCL protein or instead result from a more comprehensive and sustained defense strategy involving coordinated induction of the GCL subunit genes. In the present study, quantitative real-time PCR analysis of Gclc and Gclm expression demonstrates a clear induction of both subunits in response to oxLDL treatment. Interestingly, the induction of the modifier subunit was considerably more robust than that observed for the catalytic subunit. A survey of constitutive expression levels of both the catalytic and modifier subunits reveals that their expression levels are highly variable among tissues and do not correlate with one another. Thus, it may be that in certain tissues, the abundance of the modifier subunit is a limiting factor in GSH synthesis. This is particularly notable in light of a recent study describing an increased rate of myocardial infarction in patients with a polymorphism in the 5'-flanking region of the GCLM subunit. Patients with the GCLM promoter polymorphism had decreased plasma GSH levels, and cultured monocytes/macrophages from these individuals exhibited impaired induction of GCLM mRNA in response to treatment with pro-oxidants. It is currently unknown whether similar functional polymorphisms occur in the mouse genes. Nevertheless, these data lend support to the hypothesis that GCL subunit expression is an important determinant of GSH synthesis and that deficiencies of GSH may compromise macrophage defense mechanisms.
triggers an increase in nuclear protein binding at these sites. The increase in binding was much more dramatic for the ARE4 site in comparison to ARE3; however, constitutive binding was greater for ARE3 (data not shown). Functional studies using luciferase reporter constructs reveal a similar pattern, with ARE3 being a constitutive regulator and ARE4 responsible for both constitutive and inducible Gclc induction. These data are consistent with the findings derived from human GCLC constructs.

For the Gclm subunit, we limited our investigation to the single characterized ARE in the proximal promoter region. Our transfection studies indicate that regulatory elements mediating induction in response to oxLDL lie within the first 1.3 kb of the Gclm proximal promoter. This region is highly conserved between mouse and human, and the role of this ARE in mediating induction of human GCLM expression in response to pro-oxidants has been well described. EMSA analyses reveal that RAW 264.7 cell nuclear factors recognize and bind to this ARE in response to oxLDL. Mutation of this ARE inhibits both binding of nuclear proteins and induction of luciferase activity, thus establishing a functional role for this ARE. However, mutation of the Gclm ARE binding site does not completely attenuate induction of reporter activity in response to oxLDL, indicating that other regulatory elements might be involved as well. We also observed induced binding to consensus AP-1 oligonucleotides in RAW 264.7 cells treated with oxLDL (data not shown). Binding of nuclear factors to canonical AP-1 sites has been reported in endothelial cells exposed to oxLDL.

EMSAs implicate the transcription factors Nrf1, Nrf2, and c-jun as nuclear proteins binding to the Gclc and Gclm AREs in response to treatment with oxLDL. These transcription factors have been characterized as important regulators of ARE-driven gene expression. This was first reported by Venugopal et al., who demonstrated that overexpression of Nrf1 and Nrf2 in HepG2 cells potentiates induction of NAD(P)H:quinone oxidoreductase 1 (NQO1) and ARE-mediated expression in response to xenobiotics and antioxidants. Furthermore, transient overexpression of wild-type Nrf2 in HepG2 cells results in an increased activity of GCLC and GCLM promoter/reporter constructs. A role for the transcription factor c-jun has also been described for the induction of human GCLC ARE-mediated expression.

Although it is clear that oxLDL is a potent inducer of the expression of both the Gclc and Gclm subunits, it is not yet clear exactly which components of oxLDL are responsible for the observed activity. Copper oxidation of LDL results in formation of a large number of oxidation products derived from polyunsaturated fatty acids, phospholipids, and cholesterol, all of which could potentially be responsible for inducing Gcl subunit expression. A comprehensive analysis of the exact components responsible for inducing Gcl subunit expression awaits future studies.

In summary, we report the induction of Gcl subunit mRNAs by oxLDL in mouse RAW 264.7 cells and peritoneal macrophages. EMSAs and transfection studies with Gclc and Gclm reporter constructs demonstrate that
the observed induction involves AREs present within the \textit{Gclc} and \textit{Gclm} promoters. These data now provide a mechanistic link between oxLDL and the synthesis of GSH and could lead to potential therapies for oxidative stress–induced macrophage cell death and atherosclerotic plaque rupture.

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**References**

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