Increased Expression of Glutathione Reductase in Macrophages Decreases Atherosclerotic Lesion Formation in Low-Density Lipoprotein Receptor–Deficient Mice

Mu Qiao, Marta Kisgati, Jill M. Cholewa, Weifei Zhu, Eric J. Smart, Melanie S. Sulistio, Reto Asmis

Objective—Thiol oxidative stress leads to macrophage dysfunction and cell injury, and has been implicated in the development of atherosclerotic lesions. We investigated if strengthening the glutathione-dependent antioxidant system in macrophages by overexpressing glutathione reductase (GR) decreases the severity of atherosclerosis.

Methods and Results—Bone marrow cells infected with retroviral vectors expressing either enhanced green fluorescent protein (EGFP) or an EGFP-fusion protein of cytosolic GR (GR\textsubscript{cyto}-EGFP) or mitochondrial GR (GR\textsubscript{mito}-EGFP) were transplanted into low-density lipoprotein receptor-deficient mice. Five weeks after bone marrow transplantation, animals were challenged with a Western diet for 10 weeks. No differences in either plasma cholesterol and triglyceride levels or peritoneal macrophage content were observed. However, mice reconstituted with either GR\textsubscript{cyto}-EGFP or GR\textsubscript{mito}-EGFP—expressing bone marrow had lesion areas (P<0.009) that were 32% smaller than recipients of EGFP-expressing bone marrow. In cultured macrophages, adenovirus-mediated overexpression of GR\textsubscript{cyto}-EGFP or GR\textsubscript{mito}-EGFP protected cells from mitochondrial hyperpolarization induced by oxidized low-density lipoprotein.

Conclusion—This study provides direct evidence that the glutathione-dependent antioxidant system in macrophages plays a critical role in atherogenesis, and suggests that thiol oxidative stress-induced mitochondrial dysfunction contributes to macrophage injury in atherosclerotic lesions. (Arterioscler Thromb Vasc Biol. 2007;27:1375-1382.)

Key Words: atherosclerosis ■ glutathione ■ macrophage ■ oxidized low-density lipoprotein ■ oxidative stress
extend beyond a simple lack in reactive oxygen species-scavenging antioxidants. Lapenna et al showed that in human atherosclerotic plaque, the GSH-dependent antioxidant system is impaired. Selenium-dependent glutathione peroxidase activity was dramatically reduced, and GR activity was markedly lower in plaques than in the normal arteries. Studies in apoE-null mice showed that lipid peroxidation and atherogenesis is preceded by the depletion of GSH in the atheroma-prone aortic arch, further supporting a critical role for the GSH-dependent antioxidant in protecting against atherosclerosis. Interestingly, glutathione peroxidase-1 deficiency in mice does not appear to accelerate atherosclerosis, indicating that the disruption of the GSH-dependent antioxidant system in atherosclerotic lesions may occur at a more fundamental level, eg, the regeneration of GSH from GSSG by GR.

A large body of evidence now suggests that macrophage injury and macrophage foam cell death contribute to the development of atherosclerotic plaques. The mechanisms underlying macrophage injury in vivo are still unclear, but previously we demonstrated that the GSH-dependent antioxidant system plays a critical role in protecting macrophages from oxidative stress-induced cell injury. OxLDL, a prominent component of atherosclerotic lesions, is highly cytotoxic and believed to be a major factor in the development and progression of atherosclerosis. Studies by Darley-Usmar et al showed that depletion of cellular GSH enhances OxLDL cytotoxicity in macrophage-like cell lines, supporting a role for the GSH-dependent antioxidant system in protecting macrophages from OxLDL-induced cell death. We recently confirmed these findings in human monocyte-derived macrophages and demonstrated that OxLDL promotes thiol oxidative stress and cell injury by disrupting the GSH redox state. Our data suggest that 3 major pathways contribute to OxLDL-induced macrophage injury: (1) depletion of GSH; (2) inhibition of GR; and (3) oxidation of protein thiols. The convergence of these 3 pathways results in enhanced and sustained protein S-glutathionylation and, subsequently, in cell death.

Inhibition of GR with pharmacological inhibitors, or with siRNA directed against GR, sensitizes macrophages to OxLDL cytotoxicity, supporting a critical role for GR in protecting macrophages from thiol oxidative stress. Because OxLDL is believed to be a major factor in the development and progression of atherosclerosis, we hypothesized that protecting macrophages from OxLDL-induced cell injury by preventing the collapse of the GSH redox state would decrease the severity atherosclerosis. To test this hypothesis, we overexpressed cytosolic GR in macrophages of atherosclerosis-prone LDL-receptor–deficient (LDL-R−/−) mice. We also overexpressed GR targeted to mitochondria to examine if enhancing the regeneration of GSH from GSSG in mitochondria would protect macrophages from mitochondrial dysfunction and reduce atherosclerotic lesion formation. Our results show that enhanced expression of either cytosolic or mitochondrial GR in bone marrow-derived cells protects macrophages from OxLDL-induced mitochondrial dysfunction and reduces atherosclerotic lesion formation.

### Materials and Methods

#### Construction of Retroviral Vectors and Virus Production

Murine stem cell virus was chosen for these studies because it achieves stable high-level gene expression in hematopoietic stem cells through a specially designed 5’ long-terminal repeat from the murine stem cell virus (MSCV). The retroviral vectors were generated as follows. First, we developed a Cre-loxP-compatible acceptor vector by inserting a loxp site (Creator Acceptor Vector Construction Kit; Clontech, Mountain View, Calif) into the multi-cloning of pMSCVneo carrying the murine stem cell virus backbone (Clontech). Full-length cDNA for murine GR, which contained a mitochondrial targeting sequence (GRmt), and a truncated form of the cDNA, which lacked only the mitochondrial targeting sequence (GRcyto), were cloned in frame with the expression cassette for enhanced green fluorescent protein (EGFP). The cDNAs were kindly provided by Dr Dieter Werner (German Cancer Research Center, Heidelberg, Germany). Both forms of GR are therefore expressed as C-terminal EGFP fusion proteins (GRmt-EGFP and GRcyto-EGFP).

The sequences for EGFP, GRmt-EGFP and GRcyto-EGFP, were cloned into the Cre-loxP-compatible pDNR donor vectors (Clontech). Recombinant retroviruses expressing EGFP, GRmt-EGFP, or GRcyto-EGFP were generated in the Phoenix ECO packaging cell line (ATCC, Manassas, Va). High viruses producing cells were selected for high production by fluorescence-activated cell sorter, and viral titers were determined in NIH 3T3 cells by flow cytometry. Viral titers were 3.6×10^6 cfu/mL for EGFP, 1.1×10^7 cfu/mL for GRmt-EGFP, and 1.1×10^7 cfu/mL for GRcyto-EGFP.

#### Transduction of Bone Marrow Cells

Three days before harvesting bone marrow cells, donor mice were injected intraperitoneally with 5-fluorouracil (150 mg/kg). Bone marrow cell suspensions were isolated from C57BL/6J mice by flushing the femurs and tibias with phosphate-buffered saline containing 2% fetal bovine serum. Cells were pooled, washed, and resuspended in DMEM with 15% fetal bovine serum. Single-cell suspensions were prepared by passing the cells through a cell strainer. Cells were plated in a 60-mm culture dishes at 2×10^6/dish and incubated for 48 hours in DMEM supplemented with 15% fetal bovine serum, mr-IL-3 (10 µg/mL), mr-IL-6 (10 µg/mL), and mr-SCF (230 µg/mL). Transductions with EGFP, GRmt-EGFP, or GRcyto-EGFP retroviruses were performed by transfusing bone marrow cells into Retronectin-coated dishes (Takara, Shiga, Japan) containing 1.3 mL retroviral supernatant 15% fetal bovine serum, mr-IL-3 (10 µg/mL), mr-IL-6 (10 µg/mL), mr-SCF (230 µg/mL), and polybrene (5 µg/mL). Cells were incubated for 4 hours and the transduction procedure was repeated. After incubating cells for 18 hours, a third transduction (4 hours) was performed. Transduced cells were washed with phosphate-buffered saline before tail-vein injection. An aliquot was analyzed by flow cytometry to determine the percentage of EGFP-expressing cells after transduction with retroviruses carrying EGFP, GRmt-EGFP, or GRcyto-EGFP.

#### Mice

Female LDLR−/− recipient mice (B6.129S7-Ldlrnull/J, stock no. 002207) on a C57BL/6J background and C57BL/6J donor mice were obtained from The Jackson Laboratories (Bar Harbor, Me). All mice were maintained in a barrier facility and fed normal mouse laboratory diet. To induce hypercholesterolemia, mice were fed a diet supplemented with fat (21% wt/wt) and cholesterol (0.15% wt/wt; diet no. TD88137; Harlan Teklad) beginning at 5 weeks after bone marrow transplantation for a total of 10 weeks. All studies were performed with the approval of the University of Kentucky Institutional Animal Care and Use Committee.
Bone Marrow Transplantation
Two weeks before irradiation and bone marrow transplantation, recipient LDLR−/− mice were put on acidified water containing sulfamethoxazole (160 ng/mL) and Trimetoprim (32 ng/mL). Mice were maintained on antibiotics for 7 weeks. Before transplantation, recipient LDLR−/− mice received 2 equal doses of 4.5 Gy 3 hours apart (9 Gy total; Mark I-68 Irradiator). Bone marrow cells transfected with EGFP, GR−/−EGFP, or GR−/−EGFP retroviruses were injected into the tail vein of the irradiated recipients (n = 10 per group). Two mice in the GR−/−EGFP group died within 2 weeks after bone marrow transfer. Thus, for all experiments shown, animal numbers were 10, 10, and 8 for the EGFP, GR−/−EGFP, and GR−/−EGFP groups, respectively, unless stated otherwise.

Macrophages
Resident peritoneal cells were harvested by lavage with 5 mL of ice-cold RPMI (Gibco BRL, Carlsbad, Calif) supplemented with 2 mmol/L L-alanyl-L-glutamine (GLUTAMAX-1; Gibco BRL), 1% v/v nonessential amino acids (Gibco BRL), penicillin G/streptomycin (100 U/mL and 100 μg/mL, respectively; Gibco BRL), referred to from here on simply as culture medium. Cell viability was determined by trypan blue exclusion and was >95% in all cell preparations. Cells were plated in culture medium supplemented with 10% fetal bovine serum and cultured in a humidified atmosphere at 5% CO2. After 3 hours, nonadherent cells were removed through washing with culture medium. Human monocyte-derived macrophages were isolated and cultured as described previously.

Plasma Cholesterol and Triglycerides
Mice were fasted for 4 hours before euthanasia. After macrophages were harvested, blood was drawn by cardiac puncture. Plasma cholesterol and triglyceride levels were determined using enzymatic assay kits (Wako Chemicals, Richmond, Va).

Analysis of Atherosclerosis
After mice were euthanatized, the right atrium was removed and hearts and aortas were perfused with phosphate-buffered saline through the left ventricle. Hearts were embedded in OCT and frozen on dry ice. Aortas were fixed overnight with 4% formaldehyde in phosphate-buffered saline, dissected from the proximal ascending aorta to the bifurcation of the iliac artery, and had adventitial fat removed. For en face analysis, aortas were stained with oil red O, opened longitudinally, pinned flat onto black paper adhesive in phosphate-buffered saline, dissected from the proximal ascending aorta to the bifurcation of the iliac artery, and had adventitial fat removed. For en face analysis, aortas were stained with oil red O, opened longitudinally, pinned flat onto black paper.

Immunoprecipitation and Western Blotting Analysis
Adherent peritoneal macrophages (1×106) from each mouse were lysed at 4°C for 20 minutes in buffer A consisting of 25 mmol/L MES, pH 6.4, 150 mmol/L NaCl, 1% (v/v) Triton X-100, and 60 mmol/L octyl glucoside. Lysates from mice in each group were pooled and precleared with protein A Sepharose CL4B beads (GE Healthcare Sciences, Piscataway, NJ) suspended in buffer A. Beads were removed by centrifugation, and immunoprecipitation was performed by adding 2 μg anti EGFP antibodies (Abcam, Cambridge, Mass) and overnight incubation at 4°C. Immune complexes were precipitated with preblocked protein A Sepharose CL4B beads, washed with buffer A, and resuspended in reduced sample buffer. Proteins were separated by SDS-PAGE, transferred onto polyvinylidene fluoride membrane (Immobilon-FL; Millipore, Bedford, Mass) and detected with antibodies directed against EGFP (Cell Signaling Technologies, Boston, Mass).

Glutathione Reductase Activity Assay
Macrophage lysates were prepared with 50 mmol/L KPi, pH = 7.5, containing 1 mmol/L EDTA and 1% Triton X-100. The assay was performed at 37°C in 50 mmol/L KPi, pH = 7.5, containing 1 mmol/L EDTA, bovine serum albumin (1 mg/mL), and 333 μmol/L NADPH. The enzymatic reaction was started by adding 1 mmol/L GSSG, and absorbance was monitored at 340 nm for 15 minutes on a VERSA-max plate reader (Molecular Devices, Sunnyvale, Calif). GR from Baker’s yeast served as a standard.

Adenoviral Vectors
To control GR expression levels and prevent localization artifacts caused by overexpression, we constructed a doxycycline-controlled Tet-On adenoviral gene expression system. The adenoviral vector containing both the Tet-on transcriptional activator and the Tet-responsive element were kindly provided by Dr George Smith, Department of Physiology at the University of Kentucky. To facilitate the process of cloning of genes of interest into this large construct, we inserted a loxp cassette into the multicloning site of the vector. Sequences for EGFP, cytosolic GR−/−EGFP, and mitochondrial GR−/−EGFP were first cloned into pDONR donor vectors (Clontech) and then inserted into the adenoviral vector by linear recombination using Cre recombinase (Invitrogen, Carlsbad, Calif). Expression of the GR−/−EGFP and mitochondrial GR−/−EGFP yielded enzymatically active proteins; the latter was expressed in mitochondria, as confirmed by immunohistochemistry with antibodies directed at the mitochondrial HSP60 and by confocal microscopy (Figure 1B). Infection of macrophages with adenoviruses was performed in Opti medium (Invitrogen) supplemented with 2 mmol/L L-alanyl-L-glutamine (GLUTAMAX-1; Gibco BRL), 1% v/v nonessential amino acids (Gibco BRL), penicillin G/streptomycin (100 U/mL and 100 μg/mL, respectively; Gibco BRL), 1% sodium pyruvate, and 2% human AB serum. After 6 hours, supernatants were replaced with culture medium containing 5% human AB serum. Transgene expression was induced with doxycycline (0.01 to 0.1 μg/mL; Calbiochem, San Diego, Calif).

JC-1 Assay
Mitochondrial function was assessed with the fluorescent marker JC-1 (Invitrogen), an indicator of mitochondrial membrane potential (Δψm) and integrity. Macrophages overexpressing EGFP, GR−/−EGFP, or GR−/−EGFP were exposed to OxLDL (75 μg/mL) for 16 hours, washed, and incubated with JC-1 (5 μg/mL) for 15 minutes at 37°C. Fluorescence was measured on a SpectraMax fluorescence plate reader set to excitation wavelengths of 485 nm (JC-1 monomers) and 535 nm (JC-1 aggregates), and emission wavelengths of 530 nm (JC-1 monomers) and 590 nm (JC-1 aggregates). JC-1 monomers, which fluoresces in the green spectrum (FLmon), accumulate in mitochondria, and with increasing Δψm, form aggregates, which fluoresce in the red spectrum (FLagg). Results are expressed as fluorescence ratios FLagg/FLmon and normalized to the value of untreated control macrophages.

Statistics
Data were analyzed using ANOVA (SigmaStat; SPSS Inc.). Data were tested for use of parametric or nonparametric post hoc analysis, and multiple comparisons were performed by using the Holm-Sidak method. All data are presented as mean±SE. Results were considered statistically significant at the P<0.05 level.

Results
Increased Expressions of Mitochondrial and Cytosolic GR in Bone Marrow Cells Increase GR Activity in Resident Macrophages
Expression levels of endogenous GR protein are low in macrophages. Therefore, to determine if transgene expression...
in macrophages was maintained throughout the experiment, lysates from equal numbers of peritoneal macrophages harvested from all mice in a group were pooled and subjected to immunoprecipitation and Western blot analysis. Expressions of EGFP, GRcyto-EGFP, and GRmito-EGFP fusion protein were confirmed in the respective macrophage lysates (Figure 1A), but expression levels of GRmito-EGFP appeared to be very low (Figure 1A). Because samples had to be pooled for Western blot analysis, these data only indicated an average expression levels for the transgene in each group. To examine whether transgene expression actually increased macrophages GR activity in individual mice, we used a highly sensitive enzymatic activity assay to measured GR activity in macrophage lysates from each mouse. Macrophages from GRcyto-EGFP mice showed a 57% (*P<0.004) increase in GR activity compared with macrophages from EGFP mice, whereas GR activity was increased by 70% (*P<0.002) in macrophages from GRmito-EGFP mice (Figure 2). Our results confirm that all three transgenes were expressed throughout the duration of the experiment.

To confirm that GRmito-EGFP is targeted and expressed in macrophage mitochondria, we cloned the sequences for EGFP, GRcyto-EGFP, and GRmito-EGFP into inducible adenoviral vectors and infected human monocyte-derived macrophages with purified adenoviruses. Macrophages that were infected with viruses carrying the GRmito-EGFP gene and treated with 0.01 to 0.1 μg/mL doxycycline to induce transgene expression showed EGFP expression exclusively in the mitochondria (Figure 1B), whereas adenovirus-mediated expression of GRcyto-EGFP showed a cytosolic expression pattern (Figure 1B).
Peritoneal Macrophage Content, Plasma Total Cholesterol, and Triglyceride Levels in LDL-R−/− Mice Reconstituted With Bone Marrow Expressing EGFP, Cytosolic GRcyto-EGFP, or Mitochondrial GRmito-EGFP

<table>
<thead>
<tr>
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<th>EGFP</th>
<th>GRcyto-EGFP</th>
<th>GRmito-EGFP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Macrophage yield</td>
<td>4.9±0.5</td>
<td>5.0±0.7</td>
<td>5.4±0.7</td>
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<tr>
<td>(10⁶ cells/mouse)</td>
<td></td>
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<tr>
<td>Plasma total cholesterol (mg/dL)</td>
<td>1166±106</td>
<td>1114±52</td>
<td>922±88</td>
</tr>
<tr>
<td>Plasma triglycerides (mg/dL)</td>
<td>216±27</td>
<td>158±18</td>
<td>143±14</td>
</tr>
</tbody>
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Results are expressed as mean±SE. No statistically significant differences were observed (EGFP, n=9; GRcyto-EGFP, n=10; GRmito-EGFP, n=7).

Increased Expression of Mitochondrial and Cytosolic GR in Bone Marrow Does Not Affect Peritoneal Macrophage Content or Plasma Lipid Parameters

Resident peritoneal macrophage yields were not different between mice that had received EGFP, GRcyto-EGFP, or GRmito-EGFP bone marrow (Table). This result indicates that increased expression of GR did not appear to affect monocyte extravasation or macrophage tissue content. We also observed no statistically significant differences in plasma cholesterol levels between the 3 groups (Table). There was a trend toward lower plasma triglyceride levels in GRcyto-EGFP and GRmito-EGFP mice compared with EGFP mice. Even though the differences in plasma triglyceride levels did not reach statistical significance, we cannot rule that changes in lipoprotein composition may have contributed to the decrease in atherosclerotic lesions.

Increased Expression of GR in Bone Marrow Decreases Atherosclerosis in LDL-R−/− Mice

To determine the effect of increased cytosolic or mitochondrial GR expression in hematopoietic cells on the formation of atherosclerotic lesions, we first measured the total aortic lesion area between the proximal ascending aorta and the bifurcation of the iliac artery by en face analysis of paraformaldehyde-fixed and oil red O-stained aortas. Compared with mice that received EGFP-expressing bone marrow cells, both GRcyto-EGFP and GRmito-EGFP mice showed a 32% (P<0.009) reduction in lesion area (Figure 3A). To confirm the antiatherosclerotic effects of GRcyto-EGFP and GRmito-EGFP bone marrow cells, we also quantified lesion size in the aortic root. Lesion area was measured in 8μm sections separated by 70 μm after oil red O staining. Results are expressed as mean±SE. Two-way ANOVA showed that the difference in the mean values among the different transgenes was significant (P=0.002). Unadjusted probability values obtained by pairwise multigroup comparisons (Holm-Sidak method) were GRmito-EGFP vs EGFP: P=0.00066 (significant); GRcyto-EGFP vs EGFP: P=0.041 (not significant); and GRmito-EGFP vs GRcyto-EGFP P=0.054 (not significant).

Figure 3. (A) Analysis of atherosclerosis in LDL-R−/− mice reconstituted with EGFP, GRcyto-EGFP, or GRmito-EGFP expressing bone marrow. A, En face lesion analysis. Lesion size is expressed as percent of total intimal area. Sq symbols represent mean for each group±SE. EGFP (n=10), GRcyto-EGFP (n=9), and GRmito-EGFP (n=8). B, Lesion area in the aortic root. Lesion area was measured in 8μm sections separated by 70 μm after oil red O staining. Results are expressed as mean±SE. Two-way ANOVA showed that the difference in the mean values among the different transgenes was significant (P=0.002). Unadjusted probability values obtained by pairwise multigroup comparisons (Holm-Sidak method) were GRmito-EGFP vs EGFP: P=0.00066 (significant); GRcyto-EGFP vs EGFP: P=0.041 (not significant); and GRmito-EGFP vs GRcyto-EGFP P=0.054 (not significant).

Overexpression of Mitochondrial and Cytosolic GR in Cultured Macrophages Prevents OxLDL-Induced Mitochondrial Dysfunction

To explore potential mechanisms underlying the protective effect of cytosolic and mitochondrial GR, we examined if increased expression of GR protects macrophages from thiol oxidative stress-induced mitochondrial dysfunction. Previously we reported that atherogenic OxLDL promotes thiol oxidative stress in human macrophages by disrupting the glutathione redox buffer and that OxLDL cytotoxicity in human macrophages involves mitochondrial dysfunction. We found that stimulation of human macrophages with OxLDL for up to 16 hours promoted a slow but continuous increase in the membrane potential of macrophage mitochondria (Figure 5A). After 20 to 24 hours, coinciding with the onset of cytotoxicity, we observed a collapse of the mitochondrial membrane potential, indicating the loss of mitochondrial integrity (not shown). Addition of the peroxyl...
radical scavenger Trolox, which prevents OxLDL cytotoxicity in macrophages, prevented mitochondrial hyperpolarization induced by OxLDL, implicating OxLDL-induced peroxide formation in this process (Figure 5B). To examine if increased regeneration of GSH from GSSG also protects macrophages from OxLDL-induced mitochondrial dysfunction, we generated inducible adenoviral vectors to overexpress GR<sup>cyto</sup>-EGFP, GR<sup>mito</sup>-EGFP and EGFP in monocytederived macrophages. Doxycycline-induced expression of GR<sup>cyto</sup>-EGFP and GR<sup>mito</sup>-EGFP, but not EGFP, prevented mitochondrial hyperpolarization (Figure 5C), suggesting that increasing GR activity either in the cytosol or the mitochondria protects against OxLDL-induced mitochondrial dysfunction.

**Discussion**

In this study, we provide evidence that thiol oxidative stress contributes to atherogenesis and that protecting macrophages from (thiol) oxidative stress by strengthening their GSH-dependent antioxidant system decreases the severity of atherosclerotic lesion formation. Reconstitution of LDL-R<sup>−/−</sup> mice with bone marrow cells overexpressing either cytosolic or mitochondrial GR resulted in a significant reduction in the size of lesions found in the aortic arch and descending aorta as compared with mice reconstituted with EGFP-overexpressing bone marrow. While the protective effects of cytosolic or mitochondrial GR were identical in the aortic arch and descending aorta, only GR<sup>mito</sup>-EGFP significantly reduced the severity of atherosclerosis in the aortic root. The anti-atherosclerotic effect of GR<sup>mito</sup>-EGFP was less pronounced in this vascular bed. The reason for this region-specific difference is not clear, but it suggests that the mechanisms of thiol oxidative stress are likely to differ between the different vascular beds.

Several studies demonstrated that atherosclerotic lesions are areas of increased thiol oxidative stress. Biswas et al<sup>15</sup> observed decreased GSH synthesis in lesion-prone areas of the aortic arch from apoE-null mice that appears to precede the onset of oxidative stress. The GSH-dependent antioxidant systems also appear to be suppressed in human atherosclerotic lesions,<sup>14</sup> and exogenous supplementation with GSH has shown significant benefits on the hemodynamic parameters of patients with atherosclerosis.<sup>29</sup> Our study demonstrates that strengthening macrophages’ GSH-dependent antioxidant system by increasing their GR activity is sufficient to significantly attenuate atherosclerotic lesion formation. Plasma lipid levels were not significantly altered by the expression of the GR<sup>cyto</sup>-EGFP and GR<sup>mito</sup>-EGFP transgenes, indicating that mechanisms other than changes in plasma lipids contributed to the reduction in atherosclerotic lesions. Decreased monocyte recruitment could have accounted for reduced lesion size; however, we found no evidence that monocyte recruitment was impaired. We did not observe a reduction in the numbers of resident peritoneal macrophages in mice reconstituted with GR<sup>cyto</sup>-EGFP and GR<sup>mito</sup>-EGFP compared with EGFP-expressing mice. Furthermore, the macrophage content of lesions from these mice also did not appear to be reduced, suggesting that increasing GR activity in hematopoietic cells did not limit monocyte recruitment into the vessel wall.

We found that increasing mitochondrial GR activity in macrophages reduced lesion formation in all vascular beds examined, indicating that macrophage mitochondria might be a primary target of vascular (thiol) oxidative stress. OxLDL, which is proatherogenic and accumulates in atherosclerotic lesions,<sup>30</sup> promotes thiol oxidative stress in macrophages.<sup>22</sup> OxLDL not only depletes GSH but also inhibits GR activity and promotes protein thiol oxidation, ultimately resulting in macrophage death. Here, we demonstrated that OxLDL also promotes chronic mitochondrial dysfunction, which was prevented in macrophages overexpressing either mitochondrial or cytosolic GR. Although not shown directly, increasing macrophage GR expression in vivo may therefore protect macrophage mitochondria from thiol oxidative stress-induced injury, thereby preventing macrophage dysfunction and/or cell death and thus the progression of atherosclerotic lesions.

In support of a role for mitochondrial injury in atherosclerotic lesion formation, Ballinger et al<sup>31</sup> reported that atherosclerosis-prone apoE-null mice deficient in mitochondrial manganese superoxide dismutase exhibit increased mitochondrial damage and accelerated atherosclerosis. Our data are also in good agreement with recent findings by Oliveira et al<sup>32</sup> which suggest that a low antioxidant status of mitochondria from LDL-R<sup>−/−</sup> mice may contribute to atherogenesis. The authors provide evidence that a shift in the GSH/GSSG ratios to a more oxidized state contributes to the lower antioxidant...
Figure 5. Mitochondrial dysfunction in OxLDL-stimulated macrophages. A, Time course of OxLDL-induced mitochondrial hyperpolarization. Human monocyte-derived macrophages were treated with either OxLDL (75 μg/mL, ●) or the uncoupler p-trifluoromethoxy carbonyl cyanide phenyl hydrazone (25 μmol/L, ○) for up to 18 hours and stained with the potentiometric fluorescent dye JC-1. Mitochondrial membrane polarization was determined as the fluorescence ratios FLred/FLgreen and values within each experiment were normalized to the value obtained in untreated control macrophages as described in “Experimental Procedures.” *P<0.05 vs RPMI (t=0). B, Effect of Trolox on OxLDL-induced mitochondrial hyperpolarization. Macrophages were incubated for 16 hours either in the absence (open bars) or in the presence of OxLDL (75 μg/mL, solid bars), washed, and stained with the potentiometric fluorescent dye JC-1. The peroxyl radical scavenger Trolox (250 μM) was present during stimulation where indicated. *P<0.05 vs control (no OxLDL, white bar). C, Effect of increased cytosolic or mitochondrial GR expression on OxLDL-induced mitochondrial hyperpolarization. Macrophages were infected with adenoviruses carrying EGFP, GRcyto-EGFP, or GRmito-EGFP, and gene expression was induced with doxycycline (0.01 mg/mL, 36 hours). Cells were then incubated for 16 hours either in the absence (white bars) or in the presence of OxLDL (75 μg/mL, black bars), washed, and stained with the potentiometric fluorescent dye JC-1. Results are expressed as mean±SE from 4 independent experiments measured in triplicate. *P<0.05 vs control (uninfected, no OxLDL, white bar); **P<0.05 vs EGFP.

In summary, our studies show for the first time that increased expression of macrophage GR activity reduces the severity of atherosclerotic lesion formation, providing evidence that the GSH redox state in macrophages plays a critical role in the development and progression of atherosclerotic lesions. Our data also suggest that strategies aimed at protecting macrophages from oxidative stress-induced mitochondrial dysfunction may suppress atherosclerosis.

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Disclosures
None.

References

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Supplemental Data

**Figure I:** Representative images of oil red O-stained aortas from LDL-R\(^{-}\) mice with EGFP, GR\(^{\text{cyto-EGFP}}\) or GR\(^{\text{mito-EGFP}}\)-expressing bone marrow.