Gain and Loss of Function for Glutathione Synthesis
Impact on Advanced Atherosclerosis in Apolipoprotein E–Deficient Mice

Andrea Callegari, Yuhua Liu, Collin C. White, Alan Chait, Peter Gough, Elaine W. Raines, David Cox, Terrance J. Kavanagh, Michael E. Rosenfeld

Objective—Glutamate-cysteine ligase (GCL) is the rate-limiting step in glutathione synthesis. The enzyme is a heterodimer composed of a catalytic subunit, GCLC, and a modifier subunit, GCLM. We generated apolipoprotein E (apoE)−/− mice deficient in GCLM (apoE−/−/Gclm−/−) and transgenic mice that overexpress GCLC specifically in macrophages (apoE−/−/Gclc-Tg) to test the hypothesis that significantly altering the availability of glutathione has a measurable impact on both the initiation and progression of atherosclerosis.

Methods and Results—Atherosclerotic plaque size and composition were measured in the innominate artery in chow-fed male and female mice at 20, 30, 40, and 50 weeks of age and in the aortic sinus at 40 and 50 weeks of age. The apoE−/−/Gclm−/− mice more rapidly developed complex lesions, whereas the apoE−/−/Gclc-Tg mice had reduced lesion development compared with the littermateapoE−/− control mice. Transplantation of bone marrow from the apoE−/−/Gclm−/− and apoE−/−/Gclc-Tg mice into apoE−/− mice with established lesions also stimulated or inhibited further lesion development at 30 weeks posttransplant.

Conclusion—Gain and loss of function in the capacity to synthesize glutathione especially in macrophages has reciprocal effects on the initiation and progression of atherosclerosis at multiple sites in apoE−/− mice. (Arterioscler Thromb Vasc Biol. 2011;31:00-00.)

Key Words: antioxidants  atherosclerosis  macrophages  apo E mice  glutathione

Reduced glutathione (GSH) is a tripeptide thiol (glutamate-cysteine-glycine) that is made by most mammalian cells at up to millimolar concentrations. It is a major endogenous antioxidant and enzyme cofactor and participates in diverse cellular processes.2 The enzyme glutamate-cysteine ligase (GCL) is the rate-limiting step in the GSH synthetic pathway. GCL is a heterodimer composed of a heavy catalytic subunit (GCLC, 72.8 kDa) and a lighter modifier subunit (GCLM, 30.8 kDa).2 The 2 subunits are products of separate genes in both mice and humans.3–5 The catalytic efficiency is dramatically increased when the 2 subunits interact,6 although GCLC has catalytic activity in the absence of GCLM.6,7 Knockout of the GCLC gene is embryonic lethal, whereas GCLC heterozygote mice exhibit a 20% reduction in GSH levels.8 Reduced GCLC expression and activity has been associated with diabetes mellitus, inflammatory lung diseases, AIDS, and aging.9–11

The promoter/enhancer region of both the GCLC and GCLM genes contain a consensus antioxidant response element, and in response to changes in the cellular redox status, GCLC and GCLM expression are increased by the activation and binding of the transcription factor Nrf-2 to the antioxidant response element.12,13 We have previously shown that mouse macrophages have increased expression of both GCLC and GCLM when treated with oxidized low-density lipoprotein or homocysteine in vitro and that this increase in expression is mediated by binding of Nrf-2 to the antioxidant response element in the promoter for both genes.13,14 Increased GCLC and GCLM expression have also been reported in a variety of different cell types following treatment with other electrophiles.5,16

Depletion of GSH may play a pivotal role in cardiovascular disease. For example, polymorphisms in the 5′ flanking region of the GCLC and GCLM genes are associated with an increased risk of myocardial infarction and endothelial dysfunction.17,18 Pharmacological manipulation of GSH levels modestly alters early atherosclerotic lesion development in hyperlipidemic mice.19 The GSH content of the aorta is reduced before and during lesion development in mice,20 and GSH-dependent antioxidant enzyme expression is reduced as lesions progress.21 However, the effects of genetically altering GSH levels on advanced lesion development and composition have not been previously reported. Thus, in this study, we have evaluated the effects of GCLM deficiency that causes a dramatic systemic reduction in GSH availability22 and increased GCLC expression specifically in macrophages.
to test the hypothesis that alterations in glutathione availability, especially in macrophages, have an impact on the development and composition of advanced atherosclerotic lesions in the innominate artery and aortic sinus in chow-fed apolipoprotein E (apoE)−/− mice between 20 and 50 weeks of age.

Materials and Methods

Animals
Mice doubly deficient in apoE and Gclm (apoE−/−/Gclm−/−) were generated by breeding Gclm+/− mice on a C57Bl/6 background22 with apoE−/− mice on a C57Bl/6 background and backcrossing for more than 6 generations. Genotypes were confirmed by polymerase chain reaction (PCR), and littermate apoE−/− mice were used as wild-type controls. Mice that overexpress GCLC specifically in macrophages were generated using a construct that contained a 342-bp fragment of the CD68 gene/apolipoprotein E (apoE) promoter in addition to the CD68 gene promoter prior to the CD68 first intron, the 1920-bp full-length coding sequence for GCLC, and the bovine growth hormone polyadenylation signal. Clones were screened for correct orientation and coding sequence for GCLC, and the bovine growth hormone polyadenylation signal. Clones were screened for correct orientation and then sequenced. CBA/B6 eggs were microinjected with purified adenylation signal. Clones were screened for correct orientation and backcrossing for more than 6 generations. Genotypes were confirmed by PCR, and littermate apoE−/− mice were used as controls. Western blotting was also used to verify overexpression of GCLC in thioglycollate-elicited peritoneal macrophages isolated from the transgenic and littermate control mice (Supplemental Figure I, available online at http://atvb.ahajournals.org). All mice included in this study were fed standard mouse chow. All animal procedures were approved by the University of Washington Institutional Animal Care and Use Committee.

Genotyping Mice by PCR
DNA was isolated from mouse tail snips using the DNeasy kit (Qiagen). The mutant and the wild-type allele for GCLM and the GCLC transgene were amplified by PCR. The targeted allele for GCLM was identified by the following primer: forward 5'-CGATTTGAGGGGACCGGAAA-3'. The wild-type allele was identified by the forward primer 5'-GCCCGCTCGCCATCTCTC-3'. Both alleles shared the reverse primer 5'-GTAGTACGCCAGTCTCCCGT-3'. Analysis of the GCLC transgene used the following primers: forward 5'-TTCTCGGCTCTGTGAT-3'; reverse, 5'-CAGCCCTCCTTGGAAAGGAGG-3'.

Bone Marrow Transplantation
To determine whether a reduced or increased GSH content in macrophages would influence the progression of established atherosclerotic lesions, we performed bone marrow transplantation with bone marrow from 8-week-old male apoE−/−, apoE−/−/Gclm−/−, or apoE−/−/Gclc-Tg mice transplanted into irradiated female apoE−/− mice (the recipient mice were all obtained from the same breeding colony as the apoE−/− mice used to generate the apoE−/− GCLCTg mice). One hundred twenty 20-week-old apoE−/− females received a dose of 950 rad of whole-body x-ray irradiation. The irradiated recipients were divided into 3 groups that were injected with 1×10^7 bone marrow cells from the apoE−/−, apoE−/−/Gclm−/− or apoE−/−/Gclc-Tg male mice by tail vein injection. Atherosclerosis development and composition were analyzed at 20 and 30 weeks after bone marrow transplantation.

Mouse Perfusion and Dissection
At 20, 30, 40, and 50 weeks of age (40 or 50 weeks in the bone marrow transplantation studies), blood was collected via the retroorbital sinus, and the animals were euthanized by pharmacological overdose of a xylazine and ketamine mix (intraperitoneal). The mice were first perfused with 20 mL of PBS and then with 20 mL of fixative (4% paraformaldehyde) at physiological pressure via the left ventricle. The heart with the aortic root and the thoracic aorta and its branching vessels were dissected out intact. The ascending aorta and carotid arteries were separated from the heart and the heart was further fixed in 1% paraformaldehyde and then paraffin embedded. The innominate arteries were dissected, processed, and paraffin embedded. Five-μm-thick sections from each of the paraffin blocks were generated, and every fifth section was stained with a modified Movat pentachrome stain.

Plaque Size and Composition Analysis
The cross-sectional lesion area was determined in each Movat-stained section using computer assisted morphometric analysis (Image Pro, Media Cybernetics, Silver Spring, MD). We also tabulated the frequency of features of plaque composition in each Movat-stained section. These included the following: thin fibrous cap (defined as <3 cell layers), large necrotic core (defined as occupying >50% of the volume of the plaque), intraplaque hemorrhage (defined as the presence of red blood cells), medial enlargement/erosion (defined as the replacement of the normal media by plaque components), calcification, presence of foam cells, presence of chondrocyte-like cells, and lateral xanthomas (defined as the presence of aggregates of macrophage-derived foam cells situated on the lateral margins of the plaques). These parameters were recorded as binary outcomes, and the frequency per lesion for each animal was determined. The total number of cells in the lesions was determined by counting nuclei in each section, and the number was then normalized to the area of the lesion. All data collection was done with the operator blinded to the treatment groups.

Immunohistochemistry
Paraffin-embedded sections of the innominate artery were deparaffinized and rehydrated. The endogenous peroxidase activity was blocked by incubation with Peroxoblock (Invitrogen). Smooth muscle cells and macrophages were detected using mouse anti-smooth muscle actin (Dako) and rat anti-Mac2 (Pharmingen) according to the manufacturer’s protocols.Negative controls included an irrelevant antibody and omission of the secondary antibody.

Isolation and Stimulation of Mouse Peritoneal Macrophages
Macrophages were collected from the peritoneum of the apoE−/− gclm−/−, apoE−/−/Gclc-Tg, and apoE−/− mice 4 days after intraperitoneal injection of 2 mL of 4% thioglycollate. For details of the culturing of the peritoneal macrophages, see the supplemental material.

Caspase-9 Activity Assay
Thioglycollate elicited peritoneal macrophages from the apoE−/− gclm−/−, apoE−/−/Gclc-Tg, and apoE−/− mice were grown in 6-well plates at a density of 6×10^5 cells/cm^2 and treated with 10 mM acrolein for 6 hours. Cells were also treated with 0.5 mM L-stauroporin as a positive control for caspase activation. Caspase-9 activity was measured in 3 repeated experiments with the caspase-9 LEHD-AFC fluorometric assay kit (Biovision, Mountain View, CA) according to the manufacturer’s protocol.

Measurement of Cellular and Tissue GSH Content
The total glutathione content (nmol/mg protein of GSH+GSSG) in the peritoneal macrophages, liver, kidney, spleen, and lung was determined in a 96-well fluorescent microtiter plate assay as described previously. For details of this assay, see the supplemental material.

Measurement of Plasma Cholesterol and Triglyceride Levels
Total plasma cholesterol and triglyceride levels were measured using commercially available kits (Sigma). Lipoprotein cholesterol profiles
were generated by fast protein liquid chromatography as previously described. All data were expressed as mean±SE. Significant differences between means in serum cholesterol and triglycerides and lesion size were determined by using the Student 2-tailed t test for multiple comparisons. Frequency measures and nonnormally distributed data were analyzed using the Mann-Whitney test. Values of P<0.05 were considered statistically significant.

Results

Tissue Content of Total GSH

The levels of total GSH were measured in liver, spleen, kidney, lung, and thioglycollate-elicited peritoneal macrophages from apoE−/−/Gclm−/−, apoE−/−/Gclc-Tg and control apoE−/− mice (Table 1). There were radically reduced levels of GSH (>80%) in the liver, kidney, spleen, lung, and macrophages from the apoE−/−/Gclm−/− as compared with the control apoE−/− mice. The level of GSH in macrophages of the apoE−/−/Gclc-Tg mice was ≈3-fold higher as compared with macrophages from the control apoE−/− mice. There were no significant differences in the GSH content of the liver, kidney, spleen, lung, and from the transgenic and control apoE−/− mice.

Initiation and Progression of Atherosclerotic Plaques

The average cross-sectional area of atherosclerotic plaque in the innominate arteries was evaluated in both female and male mice at 20, 30, 40, and 50 weeks of age. In comparison with the littermate control mice at 20, 30, 40, and 50 weeks of age. In comparison with the innominate arteries of chow-fed apoE−/− mice, there were no differences in the frequency of these features were tabulated in the Movat-stained sections from each mouse. At 20 weeks of age, there were no differences in lesion composition between the apoE−/−/Gclm−/− and apoE−/− male mice. However, by 30 weeks, the apoE−/−/Gclm−/− male mice had a higher frequency of thin fibrous caps, large necrotic cores, cholesterol clefts, lateral xanthomas and foam cells, intraplaque hemorrhage, and chondrocyte-like cells (Supplemental Table I). In the apoE−/−/Gclm−/− female mice, an increased frequency of most of these features was already evident by 20 weeks of age, and there continued to be higher frequencies of a large necrotic core and cholesterol clefts at both 30 and 40 weeks of age and intraplaque hemorrhage at 30 weeks of age in the apoE−/−/Gclm−/− female mice. There were no differences in the frequency of plaque calcification in either the male or female mice at any time points (Supplemental Table I). The transplanted mice were also analyzed for features of plaque composition. At 20 weeks posttransplant, there was a higher frequency of cholesterol clefts, lateral xanthoma, and calcification in the experimental group compared with the control (Supplemental Table II).

Effects of Bone Marrow Transplantation on the Progression of Established Atherosclerotic Plaques

To determine whether the reduced or increased availability of GSH in macrophages contributes to the acceleration or the reduction in the progression of established lesions, we conducted bone marrow transplantation studies. There was about a 60% increase in lesion area in the apoE−/− mice transplanted with bone marrow from the apoE−/−/Gclm−/− mice compared with apoE−/− mice transplanted with apoE−/− bone marrow at 30 weeks posttransplant (Figure 2A). The rate of progression of the lesions was reduced by 35% at 30 weeks posttransplant when bone marrow from apoE−/−/Gclc-Tg mice was transplanted into the apoE−/− recipients (Figure 2B).

Table 1. Tissue Glutathione Content

<table>
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<tr>
<th>Tissue</th>
<th>GSH</th>
<th>ApoE−/−/Gclm−/−</th>
<th>ApoE−/−/Gclc-Tg</th>
<th>ApoE−/−/+*</th>
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<td>Macrophages</td>
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<td>Liver</td>
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<td>Kidney</td>
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<td>Lung</td>
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<td>17.64±2.46</td>
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Data are presented as means±SE. Values are nmol/mg protein. ApoE indicates apolipoprotein E; GSH, reduced glutathione.

*Littermate controls for apoE−/−/Gclm−/− mice. n=3 mice/group.

†P<0.05 vs apoE−/− mice.
**Figure 1.** Average lesion area in the innominate artery and aortic sinus. A, C, and E, ApoE−/−/Gclm−/− mice. A, Male mice at 20 weeks (ApoE−/−/Gclm−/− n=5, ApoE−/−/Gclm+/+ n=17), 30 weeks (ApoE−/−/Gclm−/− n=8, ApoE−/−/Gclm+/+ n=13), 40 weeks (ApoE−/−/Gclm−/− n=7, ApoE−/−/Gclm+/+ n=10), and 50 weeks (ApoE−/−/Gclm−/− n=9, ApoE−/−/Gclm+/+ n=4). C, Female mice at 20 weeks (ApoE−/−/Gclm−/− n=5, ApoE−/−/Gclm+/+ n=24), 30 weeks (ApoE−/−/Gclm−/− n=8, ApoE−/−/Gclm+/+ n=4), 40 weeks (ApoE−/−/Gclm−/− n=6, ApoE−/−/Gclm+/+ n=10), and 50 weeks (ApoE−/−/Gclm−/− n=5, ApoE−/−/Gclm+/+ n=20). E, Aortic sinus of female mice at 40 weeks of age (ApoE−/−/Gclm−/− n=5, ApoE−/−/Gclm+/+ n=5). B, D, and F, ApoE−/−/Gclc-Tg mice. B, Male mice at 20 weeks (ApoE−/−/Gclc-Tg n=11, ApoE−/−/Gclc-WT n=12), 30 weeks (ApoE−/−/Gclc-Tg n=7, ApoE−/−/Gclc-WT n=6), 40 weeks (ApoE−/−/Gclc-Tg n=6, ApoE−/−/Gclc-WT n=11), and 50 weeks (ApoE−/−/Gclc-Tg n=7 ApoE−/−/Gclc-WT n=13). D, Female mice at 20 weeks (ApoE−/−/Gclc-Tg n=14, ApoE−/−/Gclc-WT n=15), 30 weeks (ApoE−/−/Gclc-Tg n=15, ApoE−/−/Gclc-WT n=12), 40 weeks (ApoE−/−/Gclc-Tg n=7, ApoE−/−/Gclc-WT n=7), and 50 weeks (ApoE−/−/Gclc-Tg n=8, ApoE−/−/Gclc-WT n=18). F, Aortic sinus of male mice at 50 weeks of age (ApoE−/−/Gclc-Tg n=5, ApoE−/−/Gclc-WT n=5). Data are presented as means±SE. *P<0.05 vs controls. ApoE indicates apolipoprotein E; WT, wild-type.
higher frequency of plaques containing foam cells, whereas there was no significant difference in the females. In the female apoE/−/−Gclm-Tg mice at 40 and 50 weeks of age, there was an increased frequency of extracellular cholesterol clefts. The lesions from the mice transplanted with bone marrow from the apoE/−/−Gclm-Tg mice were also analyzed for changes in plaque composition. There were few consistent differences for both time points (Supplemental Table II). At 20 weeks posttransplant, the lesions in the apoE−/− recipients had a higher frequency of foam cells and a reduced frequency of chondrocyte-like cells and calcification compared with the control mice. At 30 weeks posttransplant, there was also an increased frequency of foam cells and a reduced frequency of large necrotic cores and chondrocyte-like cells (Supplemental Table II).

Immunohistochemistry

The presence of macrophages and smooth muscle cells in the plaques was confirmed by immunoperoxidase staining for the macrophage-specific marker Mac2 and for smooth muscle actin. There were fewer macrophages in the lesions of the apoE−/−/Gclm−/− male mice compared with age matched littermate controls at 30 weeks of age. (Figure 3A). The number of cells staining positive for smooth muscle actin increased with formation of the fibrous cap in the apoE−/−/Gclm−/− male mice at 20 weeks of age (Supplemental Figure II). The lesions in the apoE−/−/Gclm-Tg mice had more macrophage staining compared with age-matched controls at 30 weeks of age in both male and female mice (Figure 3B and 3D). The smooth muscle actin content of the lesions in the apoE−/−/Gclm-Tg was higher in the males at 20 weeks of age (Supplemental Figure II).

Reduced Lesion Cellularity and Activation of Caspase-9

There was a reduced overall cellularity of the lesions (total number of nuclei/1000 μm²) in both the male and female apoE−/−/Gclm−/− mice compared with the littermate controls at all time points (Figure 4A and 4C). There were no differences in the total cellularity of the lesions in both the male and female apoE−/−/Gclm-Tg mice compared with the littermate controls at any time point (Figure 4B and 4D). To help explain the reduced macrophage content and lesion cellularity in the apoE−/−/Gclm−/− mice, we investigated whether peritoneal macrophages from the apoE−/−/Gclm−/− mice were more susceptible to apoptosis induced by the prooxidant acrolein and whether the macrophages from the apoE−/−/Gclm−/− mice were less susceptible than macrophages from apoE−/− mice. There was an increase in caspase-9 activity (1.8-fold) in the macrophages of the apoE−/−/Gclm−/− mice following treatment with acrolein. The cells were also more susceptible to staurosporine (2.2-fold increase), and when cultured in standard medium, they showed a higher baseline caspase-9 activity (∼1.8-fold increase) than the macrophages from the control apoE−/− mice (Figure 4E). There was no difference in caspase-9 activity in the peritoneal macrophages from the apoE−/−/Gclm-Tg mice after treatment with acrolein compared with macrophages from the apoE−/− mice (Figure 4F).

Plasma Cholesterol and Triglyceride Levels

There were no differences in the total plasma cholesterol levels between the age-matched apoE−/−/Gclm−/− and apoE−/− or the apoE−/−/Gclm-Tg and control apoE−/−
mice (Table 2). There were also no differences in total plasma triglycerides. However, the analysis of the lipoprotein cholesterol profiles by fast protein liquid chromatography showed a significant difference between the apoE/H11002/H11002/Gclm/H11002/H11002 and apoE/H11002/H11002/Gclc-Tg mice at 20 weeks of age, with the majority of the cholesterol in the very-low-density lipoprotein fraction in apoE/H11002/H11002/Gclm/H11002/H11002 mice with corresponding reductions in low-density lipoprotein and high-density lipoprotein cholesterol. These differences disappeared in the apoE/H11002/H11002/Gclc-Tg mice at 50 weeks of age (Supplemental Figure IV).

In contrast, there were no differences in the lipoprotein cholesterol profiles in the apoE/H11002/H11002 mice 20 weeks following transplantation with bone marrow from the apoE/H11002/H11002/Gclm/H11002/H11002 mice (Supplemental Figure V), suggesting that the absence of GCLM in macrophages did not account for the altered profile seen in the apoE/H11002/H11002/Gclm/H11002/H11002 mice at 20 weeks of age.

**Discussion**

An association between glutathione metabolism and cardiovascular disease has been previously established,17,18,29–33 and experimental studies have provided further support for this association.20,21,34–36 For example, Rosenblat et al previously demonstrated that manipulation of GSH levels in very young apoE/H11002/H11002 mice by pharmacological interventions modestly increased or protected against early fatty streak development in the aortic sinus.19 Our current data are consistent with these previous observations and with previous in vitro and in vivo studies that show that increased GSH levels have antiatherosclerotic effects.14,15,37,38 Importantly, this study is the first to show that increasing or decreasing endogenous antioxidant levels in macrophages is sufficient to have a measurable impact on established atherosclerosis.

The apoE/H11002/Gclm/H11002 and apoE/H11002/Gclc-Tg colonies were generated several years apart and were originally derived from separate apoE/H11002 breeding stocks. Thus, with the exception of the bone marrow transplant studies where the recipient mice were all from the same colony, all comparisons were made with the separate littermate control apoE/H11002 mice. However, there were a few consistencies between the groups. For example, there were more dramatic effects of the reduced availability of GSH in the females from the apoE/H11002/Gclm/H11002 mice and more modest effects of increased GSH in the females from the apoE/H11002/Gclc-Tg mice. It is currently unclear why there were sex-dependent differences, although it is well known that female mice on a C57Bl/6 background develop larger lesions.39,40 Estrogen also plays a role in repressing the expression of antioxidant response element–dependent genes.41 These observations reinforce the point that it is important to evaluate lesions in both sexes and at multiple sites.42

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**Table 2**

<table>
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<tr>
<th>MACROPHAGE CONTENT</th>
<th>ApoE-/ Gclm-</th>
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**Figure 3.** Macrophage content of the lesions in the innominate artery. Shown is the area of Mac 2 staining in lesions in the innominate artery as a percentage of the total area of the lesion for males (A and B), and females (C and D). Sample sizes were identical to those listed in Figure 1. Data shown are the means ± SE. *P < 0.05 vs littermate control. ApoE indicates apolipoprotein E.
The up to 8-fold increase in lesion area in the innominate artery of the apoE−/−Gclm−/− mice is a much larger increase than observed with most other genetic or pharmacological interventions reported so far for apoE−/− mice. This may reflect the fundamental role of GSH in many cellular processes. The reduced availability of GSH in the apoE−/−Gclm−/− mice had the most dramatic effect at 20 and 30 weeks of age, a point at which the plaques were rapidly progressing. Initiation and progression of atherosclerotic lesions in mice is largely dependent on cellular influx and proliferation coupled with lipid uptake and connective tissue deposition. This suggests that GSH may play an essential role in regulating these processes. It is surprising, however, that the larger lesions in the apoE−/−Gclm−/− mice at 20 weeks of age, a point at which the plaques were rapidly progressing, and the reduced availability of GSH in the apoE−/−Gclm−/− mice had the most dramatic effect at 20 and 30 weeks of age, a point at which the plaques were rapidly progressing.
Fewer macrophages in the male mice at 30 weeks of age, a macrophage and smooth muscle cell content. There were increased oxidative stress. To address this possibility, we ple, may involve an increase in foam cell death due to central necrotic core, and intraplaque hemorrhage in mice are also all accelerated with the reduction of GSH.

The more rapid formation of the necrotic core, for example, may involve an increase in foam cell death due to increased oxidative stress. To address this possibility, we originally attempted to measure cell death in the plaques in all of the mice. Unfortunately, in keeping with previous reports, we could not get consistent data using the terminal deoxynucleotidyl transferase dUTP nick-end labeling assay or stain-ning for activated caspases with formalin fixed and paraffin embedded tissue. Thus, we compared the temporal changes in the overall cellularity of the plaques and in the macrophage and smooth muscle cell content. There were fewer macrophages in the male mice at 30 weeks of age, a reduced number of total cells in the lesions from all of the apoE−/−/Gclm−/− mice, and more macrophages in the lesions from some of the apoE−/−/Gclc-Tg mice, suggesting that the differences in the GSH levels may have contributed to controlling the influx of monocytes/macrophages or the turnover of the cells. This is in keeping with previous in vitro studies that have shown that depletion of GSH makes vascular cells more susceptible to prooxidant-induced cytotoxicity. It is also consistent with studies showing that alterations in macrophage glutathione content affects nuclear factor-κB activation and expression of proinflammatory cytokines that stimulate adhesion molecule expression by endothelial cells and thus recruitment of monocytes/macrophages into developing lesions.

However, protection from oxidant-induced cytotoxicity may not have contributed significantly to the modestly reduced atherosclerosis in the apoE−/−/Gclc-Tg mice, as there were no differences in the cellularity of the plaques or in the caspase-9 activity in the peritoneal macrophages. This suggests that there may be minimal oxidative stress in chow-fed apoE−/− mice. It is conceivable that there would have been more dramatic protective effects had we fed the mice a high-fat diet or induced diabetes or uremia to elevate the levels of oxidative stress. The atheroprotective effect of increasing GSH in macrophages may also be due to the role that GSH plays in maintaining redox-dependent signal transduction and protein glutathionylation, or to indirect effects mediated by other antioxidants, such as α-lipoic acid.

The current results showing that increasing the capacity of macrophages to make GSH provides some protection from lesion progression even after lesions have been established is in contrast to our recent study showing no effect on the progression of established lesions in the apoE−/− mice following dietary treatment with antioxidants. As antioxidant supplementation in humans has also not been very effective at reducing clinical events related to cardiovascular disease, our data suggest that increasing endogenous anti-oxidant production rather than supplementing with dietary antioxidants may be an effective alternative therapeutic approach for controlling the progression of atherosclerosis.

**Acknowledgments**

We thank Jerry Ricks, Charles Mahan, Kalynn Simmons, and Brent Read for expert technical assistance in the animal dissection and histology and Warren Ladiges and Carol Ware for helping to generate the Gelm−/− and Gclc-Tg mice.

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**Disclosures**

None.

**References**


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### Table 2. Total Plasma Thromboplastin and Triglycerides

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<td>20 wk</td>
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<td>585.8±14.2</td>
<td>499.7±19.8</td>
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<td>40 wk</td>
<td>523.9±38.4</td>
<td>388.5±18.2</td>
<td>619.9±29.4</td>
<td>496.3±21.4</td>
<td>648.2±66.7</td>
<td>594.7±38.2</td>
<td>614.0±25.0</td>
<td>508.5±36.3</td>
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<td>50 wk</td>
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<td>640.9±49.5</td>
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<td>634.0±29.5</td>
<td>593.5±26.9</td>
<td>631.0±25.3</td>
<td>558.2±22.1</td>
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Triglycerides

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<th>Female</th>
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<td>20 wk</td>
<td>582.6±94.4</td>
<td>408.5±59.8</td>
<td>551.4±93.0</td>
<td>342.2±38.8</td>
<td>626.0±52.4</td>
<td>399.1±31.6</td>
<td>551.4±34.2</td>
<td>410.4±36.3</td>
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<tr>
<td>30 wk</td>
<td>446.9±52.3</td>
<td>299.5±22.1</td>
<td>494.3±36.9</td>
<td>243.7±30.3</td>
<td>688.2±86.3</td>
<td>506.6±55.0</td>
<td>512.7±55.1</td>
<td>469.7±10.5</td>
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<tr>
<td>40 wk</td>
<td>474.2±47.6</td>
<td>251.0±41.9</td>
<td>503.6±47.9</td>
<td>363.9±24.3</td>
<td>523.9±38.4</td>
<td>395.5±20.1</td>
<td>497.9±45.6</td>
<td>424.8±26.0</td>
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<tr>
<td>50 wk</td>
<td>412.4±24.0</td>
<td>305.9±35.1</td>
<td>446.4±65.9</td>
<td>327.6±29.7</td>
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<td>206.2±65.9</td>
<td>467.7±75.2</td>
<td>357.6±41.3</td>
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Data are presented as the means±SE. Values are mg/dL. Sample sizes are identical to those listed for Figure 1. ApoE indicates apolipoprotein E.
Callegari et al

GCL Subunit Expression and Atherosclerosis

9


Gain and Loss of Function for Glutathione Synthesis: Impact on Advanced Atherosclerosis in Apolipoprotein E–Deficient Mice
Andrea Callegari, Yuhua Liu, Collin C. White, Alan Chait, Peter Gough, Elaine W. Raines, David Cox, Terrance J. Kavanagh and Michael E. Rosenfeld

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Expanded Materials and Methods

Isolation and stimulation of mouse peritoneal macrophages

Macrophages were collected from the peritoneum of the apo E−/-x gcml−/-, apo E−/−/Gclc-Tg, and apo E−/− mice four days after i.p. injection of 2 ml of 4% thioglycollate. Peritoneal exudate cells were harvested from the peritoneal cavity four times with 5 ml each of RPMI-1640-5% FBS. The cells were washed twice with 30 ml of ice-cold PBS after centrifugation at 250 x g at 4°C. Total cell numbers were determined with a hemocytometer after staining with Turk solution (Wako Pure Chemical Industries). Cells (3.2 x 10^6 cells/cm^2) were cultured in RPMI 1640 medium supplemented with 10% FBS (RPMI 1640–10% FBS) at 37°C in 5% CO2. After incubation for 2 h, the medium was changed and cultured in RPMI 1640–10% FBS for an additional 20–22 h.

Measurement of cellular and tissue GSH content

The total glutathione content (GSH+GSSG) in mouse peritoneal macrophages, liver, spleen and lung from 3 mice/group was determined in a 96-well fluorescent microtiter plate assay^{1,2}. Briefly, the cells or tissue aliquots were homogenized in TES/SB buffer (20 mM Tris, 1 mM EDTA, 250 mM sucrose, 20 mM sodium borate, 2 mM L-serine, PH 7.4) and an equal volume of 10% of sulfosalicylic acid was added to the homogenates, and precipitated protein was removed by centrifuging at 4 °C at 12,000 rpm for 5 min. 25 μl of the resulting
supernatant was added to 100 µl of 0.2 M NEM (n-ethylmorpholine) in 0.02 M KOH and 10 µl of tris carboxyethylphosphine (TCEP) were pipetted to 96-well flat-bottom fluorescent microtiter plate and incubated for 15 min at room temperature. To bring the solution to a pH of 12.5, 50 µl of NaOH were added, followed by 10 µl of 10 mM naphthaline dicarboxyaldheyde. After 30 min incubation in the dark at room temperature, the fluorescence was detected using 472 nm excitation and 528 nm emission (Molecular Devices, Spectra Max M2). All assays were performed in triplicate. Standard curves were run simultaneously with 0–25 nmol of GSSG per well and the GSH levels were calculated as nmole per microgram of soluble protein (nmole/mg) in the original cell extract.


### A

<table>
<thead>
<tr>
<th></th>
<th>Male</th>
<th>20 weeks of age</th>
<th>30 weeks of age</th>
<th>40 weeks of age</th>
<th>50 weeks of age</th>
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<tbody>
<tr>
<td>Foam cells</td>
<td>57.5% ± 19.2%</td>
<td>62.5% ± 11.2%</td>
<td>92.5% ± 7.5%</td>
<td>65% ± 11.5%</td>
<td>81.2% ± 11.6%</td>
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<td>Thin fibrous cap</td>
<td>3.3% ± 3.3%</td>
<td>0%</td>
<td>33.1% ± 9.9%</td>
<td>0%</td>
<td>7.1% ± 7.1%</td>
</tr>
<tr>
<td>Large necrotic core</td>
<td>0%</td>
<td>0%</td>
<td>14.7% ± 10.4%</td>
<td>0%</td>
<td>50.4% ± 13.7%</td>
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<tr>
<td>Cholesterol clefts</td>
<td>0%</td>
<td>0%</td>
<td>27.0% ± 11.4%</td>
<td>1% ± 1%</td>
<td>67.7% ± 13.8%</td>
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<tr>
<td>Lateral xanthomas</td>
<td>3.3% ± 3.3%</td>
<td>0%</td>
<td>46.9% ± 16%</td>
<td>1% ± 1%</td>
<td>47.7% ± 11.3%</td>
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<tr>
<td>Hemorrhage</td>
<td>0%</td>
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<td>30.5% ± 13.8%</td>
<td>0%</td>
<td>14.3% ± 12.1%</td>
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<tr>
<td>Medial thickening</td>
<td>50.8% ± 20.9%</td>
<td>80% ± 10.3%</td>
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<td>76.8% ± 10.6%</td>
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<tr>
<td>Chondrocytes</td>
<td>0%</td>
<td>0%</td>
<td>19.5% ± 8.1%</td>
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<td>67.9% ± 12.1%</td>
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<tr>
<td>Calcification</td>
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<td>0%</td>
<td>4.1% ± 4.1%</td>
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<tr>
<td></td>
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<td>20 weeks of age</td>
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<td>40 weeks of age</td>
<td>50 weeks of age</td>
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<tr>
<td>Foam cells</td>
<td>97.5% ± 10.9%</td>
<td>48.9% ± 7.3%</td>
<td>76% ± 12.5%</td>
<td>85.4% ± 6.1%</td>
<td>48% ± 19.6%</td>
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<td>Thin fibrous cap</td>
<td>7.5% ± 0.6%</td>
<td>1.7% ± 1.2%</td>
<td>28.3% ± 9.8%</td>
<td>29.2% ± 9.7%</td>
<td>4.8% ± 4.8%</td>
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<tr>
<td>Large necrotic core</td>
<td>7.5% ± 1.2%</td>
<td>0%</td>
<td>36.7% ± 12.3%</td>
<td>3.8% ± 2.6%</td>
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<td>10% ± 1.6%</td>
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<tr>
<td>Lateral xanthomas</td>
<td>32.5% ± 3.3%</td>
<td>0%</td>
<td>58.4% ± 15.2%</td>
<td>13.2% ± 6.2%</td>
<td>61.1% ± 15.9%</td>
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<td>Hemorrhage</td>
<td>5% ± 0.8%</td>
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<td>0%</td>
<td>37.3% ± 17.7%</td>
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</table>
Supplemental Table I. Composition of the Lesions in the Innominate Arteries.

The frequency of features of plaque composition were measured in male (A,C) and female (B,D) mice at 20, 30, 40, and 50 weeks of age. Data are presented as the mean percentage of the total Movat’s stained sections per lesion per mouse exhibiting the listed marker ± SE, p<0.05 vs littermate controls.
### Supplemental Table II. Composition of the Lesions in the Innominate Arteries Following Bone Marrow Transplantation.

The frequency of features of plaque composition were measured in female *apoE*-/recipient mice at 20 and 30 weeks post-transplant. Data are presented as the mean percentage of the total Movat’s stained sections per lesion per mouse exhibiting the listed marker ± SE, p<0.05 vs littermate controls.
Supplemental Figure Legends

Supplemental Figure I. Western Blot of GCLC Protein in Peritoneal Macrophages from apoE-/-/Gclc-Tg and apo E-/- Littermate Control Mice.


Supplemental Figure II. Smooth Muscle Cell Content of the Lesions in the Innominate Arteries.

Area of smooth muscle actin staining as a percentage of the total area of the lesions in both male (A, B) and female (C, D) mice. Data are presented as means ± SE.

Supplemental Figure III. Macrophage and Smooth Muscle Immunostaining in the Innominate Arteries.

ApoE-/-/Gclm-/- and Apo E-/- Littermate Controls (A-H). Representative examples of immunoperoxidase staining for smooth muscle actin and the macrophage-specific marker Mac2 in the innominate arteries of 40 week old mice.

Smooth muscle actin: apoE-/-/Gclm-/- male (A), apoE-/-/Gclm-/- female (B) apoE-/-/Gclm-/-/Gclm+/- male (C), apoE-/-/Gclm-/-/Gclm+/- female (D).

Mac2: apoE-/-/Gclm-/- male (E), apoE-/-/Gclm-/- female (F), apoE-/-/Gclm+/- male (G), apoE-/-/Gclm+/- female (H).
**ApoE-/-/Gclc-Tg mice and Apo E-/- Littermate Controls (I-P).** Representative examples of immunoperoxidase staining for smooth muscle actin and the macrophage-specific marker Mac2 in the innominate arteries of 30-week-old mice.

Smooth muscle actin; apoE-/-/Gclc-Tg male (I), apoE-/-/Gclc-Tg female (J), apoE-/-/Gclc-WT male (K), apoE-/-/Gclc-WT female (L).

Mac2; apoE-/-/Gclc-Tg male (M), apoE-/-/Gclc-Tg female (N), apoE-/-/Gclc-WT male (O), apoE-/-/Gclc-WT female (P).

**Supplemental Figure IV. Plasma Lipoprotein Cholesterol Profiles for ApoE-/-/Gclm-/-, ApoE-/-/Gclc-Tg, and Littermate Control Apo E-/- Mice.**

Lipoprotein cholesterol was measured in each fraction following FPLC separation of the plasma in 3 mice from each group at 20 and 50 weeks of age (A,B males; C,D females).

**Supplemental Figure V. Plasma Lipoprotein Cholesterol Profiles for Apo E-/- Mice Transplanted with Bone Marrow from ApoE-/-/Gclm-/- or Apo E-/- Littermate Control Mice.**

Lipoprotein cholesterol was measured in each fraction following FPLC separation of the plasma pooled from 7 apo E-/- mice transplanted with bone marrow from apoE-/-/Gclm-/- mice and 14 apo E-/- mice transplanted with bone marrow from littermate apo E-/- control mice. The plasma was obtained from mice sacrificed 20 weeks following bone marrow transplant.
Supplemental Figure I
Supplemental Figure II.
Supplemental Figure III.

**IMMUNOHISTOCHEMICAL ANALYSIS**

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LIPOPROTEIN CHOLESTEROL PROFILE

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Supplemental Figure IV.
Supplemental Figure V.