Deficiency of Glutathione Peroxidase-1 Accelerates the Progression of Atherosclerosis in Apolipoprotein E-Deficient Mice

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Background—We have recently demonstrated that activity of red blood cell glutathione peroxidase-1 is inversely associated with the risk of cardiovascular events in patients with coronary artery disease. The present study analyzed the effect of glutathione peroxidase-1 deficiency on atherogenesis in the apolipoprotein E-deficient mouse.

Methods and Results—Female apolipoprotein E-deficient mice with and without glutathione peroxidase-1 deficiency were placed on a Western-type diet for another 6, 12, or 24 weeks. After 24 weeks on Western-type diet, double-knockout mice (GPx-1−/− ApoE−/−) developed significantly more atherosclerosis than control apolipoprotein E-deficient mice. Moreover, glutathione peroxidase-1 deficiency led to modified atherosclerotic lesions with increased cellularity. Functional experiments revealed that glutathione peroxidase-1 deficiency leads to increased reactive oxygen species concentration in the aortic wall as well as increased overall oxidative stress. Peritoneal macrophages from double-knockout mice showed increased in vitro proliferation in response to macrophage–colony-stimulating factor. Also, we found lower levels of bioactive nitric oxide as well as increased tyrosine nitration as a marker of peroxynitrite production.

Conclusions—Deficiency of an antioxidative enzyme accelerates and modifies atherosclerotic lesion progression in apolipoprotein E-deficient mice. (Arterioscler Thromb Vasc Biol. 2007;27:850-857.)

Key Words: antioxidants ■ atherosclerosis ■ nitric oxide

Oxidative stress is defined as an imbalance between the production and degradation of reactive oxygen species (ROS). Enzymatic inactivation of ROS is achieved mainly by superoxide dismutases, catalase and the glutathione peroxidases. Indeed, glutathione and the glutathione peroxidases constitute the principal antioxidant defense system in mammalian cells. Glutathione peroxidase-1 (GPx-1), the ubiquitous intracellular form and key antioxidant enzyme within many cells, including the endothelium, consumes reduced glutathione to convert hydrogen peroxide to water and lipid peroxides to their respective alcohols. It also acts as a peroxynitrite reductase. Because of its major role in the prevention of oxidative stress, GPx-1 may be an important antiatherogenic enzyme. In fact, we have recently shown in patients with coronary artery disease that a low activity of red blood cell GPx-1 is associated with an increased risk of cardiovascular events independently from traditional risk factors or atherosclerosis. A mouse model of GPx-1 deficiency is available. These animals appear healthy and are fertile. However, a recent in vitro study showed increased cell-mediated oxidation of low-density lipoprotein (LDL) in this model. Furthermore, GPx-1 deficiency causes endothelial dysfunction in mice that is aggravated by hyperhomocysteinemia. GPx-1 deficiency is accompanied by increased periadventitial inflammation, neointima formation, and collagen deposition surrounding the coronary arteries. GPx-1 activity is decreased or absent in carotid atherosclerotic plaques, and the absence of GPx-1 activity in atherosclerotic lesions has been linked to the development of more severe lesions in humans. Recently, t’Hoen et al reported that the expression of several antioxidant enzymes including GPx-1 was increased in the aortic arch of apolipoprotein E-deficient (ApoE−/−) mice in the period preceding lesion formation, and that their expression decreased at the time when lesion formation became apparent, whereas Biswas et al suggested that decreased glutathione synthesis as well as reduced

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transcription and activity of GPx-1 precede lipid peroxidation and detectable atheroma in these mice.16 Despite this plethora of both experimental and clinical data linking GPx-1 deficiency to cardiovascular pathology, no direct evidence has yet established a role of GPx-1 during atherogenesis. Recently, de Haan et al17 reported that in C57BL/J6 mice GPx-1 deficiency did not accelerate atherosclerotic lesion development after 12 and 20 weeks on a high-fat diet. Since we obtained the same result in GPx-1-deficient (GPx-1−/−) C57BL/J6 mice even after 1 year on a high-fat diet supplemented with cholate (data not shown), we extended our analysis to the commonly used ApoE−/− mouse model by crossbreeding GPx-1−/− mice with this strain.

Methods

Mice
GPx-1−/− ApoE−/− mice (generously provided by Ye-Shi Ho, Department of Biochemistry, Wayne State University, Detroit, Mich) were bred by generating F2 hybrids from the ApoE−/− and GPx-1−/− parental strains. The GPx-1−/− ApoE−/− strain could then be propagated successfully by in crossing. Genotype determination was performed as described (please see http://atvb.ahajournals.org).13

Induction of Atherosclerosis
At 7 to 8 weeks of age, female ApoE−/− as well as GPx-1−/−ApoE−/− mice were placed on a Western-type diet (WTD) for another 6 weeks (5 GPx-1−/−ApoE−/− and 5 control ApoE−/− mice), 12 weeks (16 GPx-1−/−ApoE−/− and 16 control ApoE−/− mice), or 24 weeks (12 GPx-1−/−ApoE−/− and 15 control ApoE−/− mice). The WTD contained 21% (wt/wt) fat and 0.15% (wt/wt) cholesterol (snssf; Spezialdiäten GmbH, Soest, Germany). Mice were kept in accordance with standard animal care requirements, housed 4 to 5 per cage, and maintained on a 12-hour light–dark cycle. Water and food were given ad libitum.

Lipoprotein Analysis and Blood Pressure Measurements
For detailed description of quantitative cholesterol and triglyceride analyses, fast protein liquid chromatography gel filtration of pooled plasma samples as well as blood pressure measurements with a computerized tail-cuff system (please see http://atvb.ahajournals.org) were performed.

GPx-1 Activity
GPX-1 activity was determined in washed red blood cells obtained immediately after sampling from whole blood anticoagulated with EDTA. Hemolyzed cells were stored frozen for up to 1 week. Enzyme activity was measured as described, with minor modifications (Ransel; Randox, Crumlin, UK).18

Tissue Preparation and Quantitative Morphometry of Atherosclerotic Lesion Development
Mice were euthanized by exposure to carbon dioxide. Peritoneal cavities were opened and the cadavers fixed in 4% buffered formaldehyde. Hearts and aortas were resected en bloc down to the iliac bifurcation and carefully cleaned of perivascular adipose tissue under a dissection microscope (Leica MZ6; Leica, Bensheim, Germany). The aortic arch and the rest of the aorta from the arch to the iliac bifurcation were separated. Longitudinal sections of the aortic arch were stained with trichrome and computer-assisted (Image Pro Discovery; Media Cybernetics, Silver Spring, Md) measurement of plaque size was performed as described previously19 (supplemental Figure I, please see http://atvb.ahajournals.org).

Immunohistochemical and Histochemical Analyses
Immunostaining of murine tissues with the murine MAbs was performed using the Vector M.O.M. immunodetection kit (Vector Laboratories, Burlingham, Calif; please see http://atvb.ahajournals.org). The following antibodies and dilutions were used: murine monoclonal IgM E06 (1:500) binding the phosphorylcholine headgroup of oxidized but not native phospholipids, rabbit anti-murine JE (MCP-1; 1:100), murine monoclonal IgG NA59 (1:100) binding 4-hydroxynonenal lysine-epitopes, rabbit anti-nitrotyrosine (1:500; Upstate, Dundee, UK), and murine anti-smooth muscle α-actin (1A4, 1:100; Sigma, St Louis, Mo). Collagen content was analyzed by picrosirius red and polarized light microscopic imaging. Percent-positive area for immunohistochemical or picrosirius red staining of the inner aortic arch intima (lesser curvature) was quantified by Photoshop-based image analysis as described.20,21 Briefly, pixels with similar chromogen characteristics were selected with the “magic wand” tool and the “select similar” command, and the ratio of the positively stained area to the total lesion area studied was calculated with the “histogram” command in Photoshop. For details of the procedure for determination of MCP-1 immunostaining intensity please see http://atvb.ahajournals.org. All quantitative morphometric and immunohistochemical data were collected independently by 2 experienced operators blinded to the mice genotypes.

Determination of ApoB IgG and IgM Immune Complexes (IgG and IgM IC/apoB) and Autoantibodies to Oxidized LDL
The IC/apoB in mouse plasma were assessed after 12 and 24 weeks on the WTD with a “sandwich” chemiluminescence immunoassay as previously described.22,23 The levels of IgG and IgM autoantibodies binding to malondialdehyde (MDA)–LDL and copper–oxidized LDL (low-density lipoprotein) were determined by chemiluminescence-based enzyme-linked immunosorbent assay as described.24 MDA-LDL and copper–oxidized low-density lipoprotein was generated as previously described.25,26 Mice sera were diluted 1:200.

Oxidative Fluorescent Microtopography and Detection of ROS Formation Using Diogenes-Enhanced Chemiluminescence
Superoxide was detected in situ using dihydroethidium fluorescence as described recently.27 For superoxide and hydrogen peroxide detection, please see http://atvb.ahajournals.org.

Isolation of Peritoneal Macrophages
Mouse peritoneal macrophages were prepared from GPx-1−/−ApoE−/− mice by intraperitoneal injection of 1 mL 3% thioglycollate (Merck, Darmstadt, Germany). After 4 days, cells were harvested by intraperitoneal lavage with 7 mL DMEM and centrifuged for 5 minutes at 1250 rpm. The pellet was resuspended in DMEM with 10% fetal calf serum and plated in bacterial dishes. After incubation for 4 hours, nonadherent cells were removed.

Proliferation Assay
The thioglycollate-elicited macrophages were incubated for 4 days with macrophage–colony stimulating factor (10 ng/mL; PeproTech, London, UK). Cells were detached with Accutase (PAA Laboratories, Pasching, Austria) and plated in a 96-well plate (2.5 × 104 cells/well). Cells were incubated again with macrophage–colony stimulating factor and BrdU for another 16 hours, and the proliferation assay (Roche, Mannheim, Germany) was performed according to the manufacturer’s instruction. Briefly, after fixation and permeabilization of the cells, the incorporated BrdU was detected by an anti-BrdU–POD antibody followed by incubation with Luminol. The amount of bound antibody was quantified by determination of relative light units with a chemiluminescence plate reader (Fluoroscan; Thermo Labsystems, Waltham, Mass).
TUNEL Assay
Thioglycollate-elicited peritoneal macrophages were plated in 16-well chamber slides (1×10^5 cells/well) in DMEM supplemented with 10% fetal calf serum. After 24 hours, nonadherent cells were removed and the resident macrophages were incubated for another 7 days without additional stimuli. Staining of apoptotic cells was performed with the in situ cell death staining kit (Roche, Mannheim, Germany) according to the manufacturer’s instructions. Percentage of apoptotic cells was evaluated by counting the stained cells/1×10^5 cells seeded.

Reporter Cell Assay for Determination of Aortic Nitric Oxide Production
Aortic nitric oxide production was bioassayed by determination of the cGMP content in RFL-6 rat lung fibroblasts as reporter cells (please see supplemental materials).

Statistical Analyses
Data were analyzed by SPSS 12.0 for Windows (SPSS Inc). Most of the outcome parameters used in this study did not follow a normal distribution as judged by Shapiro-Wilk tests, so statistical analyses were performed with Mann-Whitney U tests. Except for plasma lipid contents and blood pressure measurements (mean±SD), data in the text are presented as median and interquartile range. Data in the Figures are presented as boxplots with median, interquartile range, minimum, and maximum. Differences were considered significant when P<0.05.

Results

Generation of GPx-1^-/- ApoE^-/- Mice
To study the role of GPx-1 in atherogenesis, we generated double-knockout (GPx-1^-/- ApoE^-/-) mice by crossing GPx-1^-/- with ApoE^-/- mice, both on the C57/BL6 background (please see supplemental materials).

GPx-1 Deficiency Does Not Affect Serum Lipoproteins or Systolic Blood Pressure
The WTD significantly increased plasma total cholesterol but not triglyceride levels in both GPx-1^-/- ApoE^-/- mice and control ApoE^-/- mice compared with baseline values with no significant differences between the 2 strains after 6, 12, and 24 weeks, respectively (supplemental Figure IIA). Furthermore, fast protein liquid chromatography analysis of lipoproteins showed that disruption of GPx-1 gene function did not affect lipoprotein distribution in mice consuming the atherogenic diet (supplemental Figure IIB).

Also systolic blood pressure was similar after 6 weeks on a WTD (118±4.4 mm Hg in GPx-1^-/- ApoE^-/- mice [n=4] versus 114.6±2.7 mm Hg in ApoE^-/- mice [n=3]).

Atherosclerosis Lesion Progression
Compared with control ApoE^-/- mice (n=15), double-knockout mice (GPx1^-/- ApoE^-/-, n=12) developed significantly more atherosclerosis after 24 weeks on the WTD as indicated by plaque area en face of the aorta from the arch down to the iliac bifurcation (Figure 1A and 1B). This difference was mainly caused by significantly more atherosclerosis in the distal part of the aorta from the diaphragm down to the iliac bifurcation (P<0.01, data not shown). A similar, albeit statistically not significant, trend could already be observed as early as after 12 weeks on the WTD (Figure 1A), but not yet after 6 weeks on the WTD (% plaque area, median and interquartile range: GPx1^-/- ApoE^-/-, 0.7/0.65; ApoE^-/-, 1.1/1.0). Taken together, these data indicate that GPx-1 deficiency accelerates atherosclerotic lesion development in ApoE^-/- mice.

Phenotypic Analysis of Atherosclerotic Lesions
Quantification of the maximal area and thickness (median/interquartile range) of the inner aortic arch intima (lesser curvature) revealed no significant differences in control mice compared with double-knockout mice after 6 weeks on the WTD (area, 446.37±257.210 μm² versus 184.36±185.06 μm²; thickness, 298/380 μm versus 203/167 μm). The relatively high medians and interquartile ranges are attributable to extreme outliers and high interindividual vari-
In contrast, consistently, albeit not significantly, less atherosclerosis was observed in control mice compared with double-knockout mice after 12 weeks (area, 115.962/114.594 \( \mu m^2 \) versus 128.633/87.096 \( \mu m^2 \); thickness, 215/151 \( \mu m \) versus 237/173 \( \mu m \)) or 24 weeks on the WTD (area, 382.551/182.114 \( \mu m^2 \) versus 416.530/71.370 \( \mu m^2 \); thickness, 328/201 \( \mu m \) versus 334/100 \( \mu m \)). Furthermore, the histomorphological aspect of lesions in double-knockout mice differed markedly from lesions in control mice in terms of plaque composition. At 12 weeks on the WTD, atherosclerotic lesions in ApoE\(^{-/-}\) mice contained significantly less macrophages (area covered by macrophages; Figures 2A and 3A, left panels) and significantly more collagen (area covered by collagen; Figures 2C and 3C, left panels) than lesions in double-knockout mice.

Figure 2. Phenotypic analysis of atherosclerotic lesions after 12 (left panels) and 24 weeks (right panels) on the WTD. Atherosclerotic lesions of the inner aortic arch intima (lesser curvature) of GPx-1\(^{-/-}\)ApoE\(^{-/-}\) and ApoE\(^{-/-}\) mice were quantified for macrophages (A), smooth muscle cells (B), and collagen (C). Percent-positive area for macrophages, smooth muscle cells, and collagen was quantified by Photoshop-based image analysis (see also Figure 3). Data are presented as boxplots with median, interquartile range, minimum, and maximum.

Figure 3. Representative examples of atherosclerotic lesion composition after 12 (left panels) and 24 weeks (right panels) on the WTD. Atherosclerotic lesions of the inner aortic arch intima (lesser curvature) of GPx-1\(^{-/-}\)ApoE\(^{-/-}\) and ApoE\(^{-/-}\) mice were stained with trichrome (A) (for quantification of macrophages), mouse anti-smooth muscle \( \alpha \)-actin (1A4) (B) (for quantification of smooth muscle cells), and picrosirius red with subsequent polarization (C) (for quantification of collagen). Percent-positive area for macrophages (*), smooth muscle cells (brown), and collagen (yellow, green, orange, and red polarized color) were quantified by Photoshop-based image analysis (see also Figure 4). The lumen is to the upper left corner. The demarcation between intima and media is indicated by an arrowhead. C. Note that the adventitial tissue (*) also polarizes after picrosirius red staining (internal positive control). Magnification \( \times 64 \).
more, a slight but yet not significant increase in the amount of smooth muscle cells could be observed (area covered by smooth muscle cells; Figures 2B and 3B, left panels). At 24 weeks on the WTD, the difference in the amount of smooth muscle cells in double-knockout mice was highly significant (Figures 2B and 3B, right panels). Macrophages and collagen content were not significantly different at this time point (Figures 2A, 2C, 3A, 3C, right panels). Collectively, these data indicate that GPx-1 deficiency leads to increased cellularity with a higher relative number of macrophages in early and a higher relative number of smooth muscle cells in advanced atherosclerotic lesions when compared with control ApoE−/− mice.

Superoxide Formation in the Aortic Wall and Detection of ROS Formation in Aortic Vessel Segments, Heart Mitochondria, and Membrane Fractions

We assessed superoxide formation in cryosectioned aortic rings after 6 weeks on a WTD by means of dihydroethidium-derived fluorescence. Staining of aortic sections with dihydroethidium revealed a marked (50%) increase in vascular superoxide in GPx-1−/−ApoE−/− mice compared with control ApoE−/− mice. This increase was observed primarily in the endothelium, but less pronounced in the intima and adventitia (Figure 4). Furthermore, detection of ROS formation in aortic vessel segments, heart mitochondria, and membrane fractions point toward increased oxidative stress in GPx-1−/−ApoE−/− mice because of activation of mitochondria and NADPH oxidases or because of impaired antioxidative defense mechanism in the GPx-1−/− animals (supplemental Figure III).

Influence of GPx-1 Deficiency on Lipoprotein Oxidation

We did not find evidence for an increased lipoprotein oxidation in GPx-1−/−ApoE−/− mice as determined by both immunohistochemical quantification of oxidized LDL and measurement of ApoB IgG and IgM immune complexes and autoantibodies to oxidized LDL (supplemental Figure IV).

Proliferation, Apoptosis, and Recruitment of Monocytes

Next, we investigated whether proliferative activity and/or less apoptosis of monocyte-derived macrophages might account for the increased cellularity of early atherosclerotic lesions in GPx-1−/−ApoE−/− mice. The proliferation rate of mouse peritoneal macrophages was investigated with a BrdU-based chemiluminescence assay. After stimulation with macrophage–colony-stimulating factor macrophages from GPx-1−/−ApoE−/− mice showed significantly more BrdU incorporation than macrophages from ApoE−/− control mice (Figure 5A). Apoptosis of mouse peritoneal macrophages was investigated with a terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling assay. After cultivation for 7 days without additional stimuli, percentage of apoptotic cells was not significantly different in control mice compared with double-knockout mice (Figure 5B).

These results indicate that increased proliferative activity rather than diminished apoptotic rates of macrophages contribute to the increased cellularity of early atherosclerotic lesions in GPx-1−/−ApoE−/− mice. Furthermore, we did not find evidence for increased expression of MCP-1 in lesions of
GPx-1−/− ApoE−/− mice, that might account for increased recruitment of monocytes/macrophages into the lesions (supplemental Figure V).

Vascular Nitric Oxide Production and Protein Nitration

The bioassay of aortic nitric oxide production indicated that less bioactive nitric oxide is generated in response to acetylcholine in aortae of double-knockout mice compared with control mice. Likewise, the observation of extensive protein nitration in atherosclerotic lesions of double-knockout mice suggests that peroxynitrite is produced and may be involved in the different evolution of the lesions in control and double-knockout mice (supplemental Figure VI).

Discussion

The present study on atherosclerosis development in GPx-1−/− ApoE−/− mice has provided several key results. Atherosclerotic lesion development is more rapid in GPx-1−/− ApoE−/− double-knockout mice with larger lesions after 12 (not significant) and 24 weeks on a WTD compared with ApoE−/− mice. The lesions are more cellular with an increase in macrophage content in early lesions and an increase in smooth muscle cell content in advanced lesions. As expected, arterial walls of double-knockout mice contain more ROS than tissue from controls. Consistent with the previously reported endothelial dysfunction in GPx-1−/− mice,11–13 aortic tissue from double-knockout mice produced less bioactive nitric oxide in response to acetylcholine than that of ApoE−/− control mice. Somewhat unexpectedly, we could not find consistent evidence of increased lipoprotein oxidation in GPx-1−/− ApoE−/− mice by immunohistochemistry as well as determination of apoB-immune complexes and autoantibodies against oxidized LDL. Taking into account the inverse association of GPx-1 activity and the risk for cardiovascular events in humans with coronary artery disease,8 these results provide further strong evidence that GPx-1 plays an important antiatherogenic role in the arterial wall.

The accelerated development of atherosclerosis in the present study is in contrast to recent observations in simple GPx-1−/− mice, which have no enhanced atherosclerotic lesion development.17 One should keep in mind though, that lesion development in fat fed C57BL/6 mice is very discrete and confined to the aortic root. Furthermore, this model is associated with severe nonvascular pathology (eg, hepatic) caused by the extreme diet modification. Therefore, it has been generally abandoned for atherosclerosis research after the availability of hyperlipidemic ApoE−/− and LDL-receptor−/− mice.

Besides the increased lesion size in double-knockout mice, another striking difference between the 2 genotypes in atherosclerotic lesion development relates to histomorphological composition in the aortic arch lesion. At 12 weeks on the WTD, lesions in the aortic arch were particularly rich in macrophages. GPx-1−/− ApoE−/− mice showed a lesional macrophage content that was approximately twice that of corresponding lesions in ApoE−/− mice (Figure 4A). This increase in macrophage cellularity was paralleled by a comparative decrease in extracellular matrix as determined by the collagen content (Figure 4C). At 24 weeks on the WTD, lesions in the aortic arch were much more advanced with smooth muscle cells being the predominant cell type. At this time point, the relative number of smooth muscle cells was significantly higher in GPx-1−/− ApoE−/− mice when compared with ApoE−/− mice with no concomitant increase in collagen content, which is surprising at first glance (Figure 4B).

However, this latter observation is in line with recent data demonstrating that increased oxidative stress activates matrix metalloproteinases and decreases fibrillar collagen synthesis in rat cardiac fibroblasts28 and, vice versa, overexpression of GPx attenuates matrix metalloproteinase-9 zymographic and protein levels in mouse myocardium.29

This propensity toward more cellular atherosclerotic lesions of GPx-1−/− ApoE−/− mice was accompanied by severely impaired disposal of ROS in mitochondria. Whereas severe oxidative stress is cytotoxic, mild oxidative stress may stimulate cell proliferation, as shown recently in various cell types including vascular smooth muscle cells.30–33 Proliferation of vascular smooth muscle cells is a hallmark of atherosclerosis development. Furthermore, many data support the concept that also macrophages in early lesions may derive, at least in part, from local proliferation, especially under circumstances of enhanced oxidative stress.34 The combination of hypertension and hyperlipidemia in LDL-receptor−/− mice with hyperglycemia and further oxidative stress by glucose-oxidized LDL has recently been shown to induce macrophage proliferation in vascular lesions.35 We could show that GPx-1 deficiency increases proliferation of macrophages in vitro in response to macrophage–colony-stimulating factor. In contrast, GPx-1 deficiency obviously does not influence the extent of apoptosis of macrophages in vitro or MCP-1 protein expression in vivo. Therefore, we propose that increased cellularity of early atherosclerotic lesions in GPx-1−/− ApoE−/− mice is mainly caused by increased proliferative activity rather than diminished apoptotic rates or increased recruitment of monocytes/macrophages into the lesions.

The data obtained in GPx-1−/− ApoE−/− mice add further information to the role of antioxidant defenses in atherosclerosis. Antioxidant enzymes have been the topic of many investigations in experimental animals and epidemiologic studies in humans. In mouse models of atherosclerosis, data are available on the 3 superoxide dismutases (SOD), catalase, and glutathione peroxidase. It is remarkable that neither overexpression of copper, zinc–SOD, or of endothelial cell–SOD protects against atherosclerosis in ApoE−/− or LDL-receptor−/− mice.36–38 Accordingly, deletion of endothelial cell–SOD is not proatherogenic.39 In contrast to these models of atherosclerosis, neointima formation in balloon injury models has been reported to be inhibited by overexpression of endothelial cell–SOD.40 Because homozygous Mn–SOD deficiency leads to severe pathology and death soon after birth, only mice with heterozygous Mn–SOD deficiency have been analyzed for atherosclerosis development. In these mice, there was an increase in branchpoint lesions observed in the aorta, interpreted as interaction of high shear-stress with the genotype. However, only 4 animals per group were analyzed and no data on the overall size of lesions were presented.41 In
of oxidative stress in atherogenesis and may eventually redirect clinical interest toward the development of effective preventive interventions in patients at risk for cardiovascular disease.

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Disclosure
None.

References

summary, the SODs with the possible exception of Mn-SOD apparently do not protect hyperlipidemic mice against atherosclerosis. Similarly, also, inactivation of NADPH oxidase (with a significant reduction in vascular superoxide generation) had no protective effect.42,43 Finally, no association of red blood cell copper-zinc-SOD activity with cardiovascular risk was observed by us in the same study that revealed the inverse association of GPx-1 activity and cardiovascular risk.5

While the role of SODs in atherogenesis has been surprisingly minor, it has been shown that overexpression of catalase is protective in a hyperlipidemic mouse model.38 Like glutathione peroxidases, catalase inactivates H2O2. Even though definitive experimental evidence is missing to date, these data implicate that H2O2 may be relatively more important in atherogenesis than superoxide anion. This may be related to the fact that the latter is very short-lived (also in the absence of SOD) and contributes to the generation of H2O2. It is conceivable that as long as H2O2 is effectively converted to water, minor changes in the production and half-life of superoxide outside mitochondria may not be critical for atherogenesis. Aside from these considerations, it is well-established that superoxide and H2O2 induce different cellular responses, eg, by different effector mechanisms in transcriptional regulation.33,44

An intriguing aspect of the increased lesion size relates to the reduction of antioxidant enzymes including GPx-1 in the aortic arch of ApoE−/− mice before or at least when lesions develop.15,16 Even though these 2 studies come to slightly different results, this may be interpreted as indicating that GPx-1 and other antioxidant enzymes cannot be critical for atherosclerotic lesion development, because they are no longer expressed at high levels when lesions developed. It should be noted, though, that there is definitely remaining GPx-1 activity at all stages of atherosclerotic lesions in the aortic arch. This could provide some protection in the ApoE−/− mouse, which is lost in the double-knockouts. Furthermore, in the early phase of lesion development, when GPx-1 expression has been reported to be normal in one study, there is a significant difference in lesion composition toward a more macrophage-rich atheroma in GPx-1−/− ApoE−/− mice. Previous work has already demonstrated that GPx-1 was expressed at higher level in the descending aorta than in the aortic arch in Apo E−/− mice from the age of 12 weeks onwards.15 The substantially higher residual expression of GPx-1 in atherosclerotic lesions of the descending aorta would indicate that the difference in enzyme activity between ApoE−/− and GPx-1−/− ApoE−/− is more pronounced in this part of the aorta. This observation might explain the larger difference in lesion development observed in the abdominal aorta after 24 weeks.

Our knowledge concerning the contributions of different ROS and their reaction products to atherogenesis is still incomplete. This fact is impressively underscored by the surprising but notorious lack of any true preventive effects of different antioxidant treatments in large clinical trials.45,46 However, the results from different genetically modified mouse models show that modification of antioxidant defense systems may indeed add important clues to our understanding...


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Methods

Mice and Genotype Determination

Laboratory mice were maintained at the Central Laboratory Animal Facility of the University of Mainz under strict SPF conditions. C57BL/6J-ApoE<sup>tm1Unc</sup> knockout mice (ApoE<sup>−/−</sup>) backcrossed to the C57BL/6 strain for 10 generations were purchased from the Jackson Laboratory (Bar Harbor, ME, strain No 2052). GPx-1<sup>−/−</sup> mice backcrossed to C57BL/6 were generously provided by Ye-Shi Ho.<sup>1, 2</sup> Both individual knockout mutants were effectively propagated by incrossing. Heterozygous GPx-1<sup>+/−</sup> mice were identified by having both a 509-bp and a 293-bp PCR product. Homozygous GPx-1<sup>−/−</sup> mice were identified by an exclusive 509-bp PCR product while wild type mice were identified by an exclusive 293-bp PCR product. The ApoE knockout and wild type alleles were discriminated as described by the Jackson Laboratory.

Lipoprotein Analysis, Gel Filtration Chromatography of Total Plasma and Blood Pressure Measurements

Murine sera were diluted 1:3 before quantitative cholesterol and triglyceride analyses. Quantitative cholesterol determinations were conducted using a colorimetric assay (CHOD-PAP, Roche Diagnostics, Mannheim, Germany). Triglycerides were determined by quantifying free glycerine originating from hydrolytic cleavage (GPO-PAP, Roche Diagnostics). Pooled plasma samples from 6 mice (100 µl) were subjected to fast protein liquid chromatography (FPLC) gel filtration using a Superose 6 column (Amersham Biosciences, Piscataway, NJ). Elution was performed at a flow rate of 0.5 ml/min. 60 fractions (0.5 ml) were collected, and cholesterol concentrations were determined enzymatically (see above). Systolic blood pressures (BP) were measured with a computerized tail-cuff system using a ML125 NIBP (Non-Invasive Blood Pressure) Controller in conjunction with a PowerLab system.
(ADInstruments, Spechbach, Germany). After 6 weeks on a WTD, mice (n = 3-5) were trained for three days to be acclimatized to the measurement protocol. Then, BP measurement were performed at 30°C for 3 consecutive days. 9 measurements were obtained and averaged for each mouse. This non-invasive method of measuring blood pressure correlates well with intra-arterial measurements in normotensive and hypertensive mice.3

Tissue Preparation and Quantitative Morphometry of Atherosclerotic Lesion Development

The maximal area and thickness of the inner-aortic-arch intima (lesser curvature) of each mouse were used to compute averages per group. The rest of the aortae from the arch down to the iliac bifurcation was opened longitudinally and stained with freshly prepared Sudan IV as previously described.4 Sudan stained atherosclerotic lesions en face were then quantified using Photoshop-based image analysis (Version 8.0.1, Adobe Systems Inc., San Jose, CA) as described.4

Immunohistochemical and Histochemical Analyses

Serial 5 µm-thick longitudinal sections of the paraffin-embedded aortic arch were deparaffinized in xylene. All slides were treated with 3% H₂O₂ to block endogenous peroxidase activity. Slides were incubated consecutively with 5% normal serum to block non-specific binding, primary antibody for 1 hour, biotin-conjugated secondary anti-mouse or anti-rabbit antibody for 30 minutes and avidin-biotin-peroxidase reagent for 45 minutes at room temperature. The reaction products were revealed by immersing the slides in diaminobenzidine tetrachloride to give a brown reaction product. The slides were then counterstained with hematoxylin and mounted. Negative controls included replacement of the primary antibody by irrelevant isotype-matched antibodies.

Determination of MCP-1 immunostaining intensity was performed by Photoshop-based image analysis as described5 with slight modifications. After selecting pixels with similar chromogen

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characteristics with the “magic wand” tool and the “select similar” command, the image was transformed to 8-bit grayscale. An optical density plot of the selected area was generated using the “histogram” tool in the “image” menu. The mean staining intensity (in arbitrary units, AU) was recorded. Subsequently, the background of the lesion area was selected using the “inverse” tool in the “select” menu, and immunostaining was quantified again using the “histogram” tool in the “image” menu. Immunostaining intensity was then calculated as the difference between MCP-1 immunostaining and background immunostaining and was designated immunohistochemical index with arbitrary units (AU).

Detection of Reactive Oxygen Species (ROS) Formation Using Diogenes™ Enhanced Chemiluminescence

The Diogenes™ cellular chemiluminescence enhancement system (National Diagnostics Inc., Atlanta, USA) for superoxide and hydrogen peroxide detection was prepared according to the manufacturer’s instructions. Aortic vessel segments, isolated heart mitochondria and membrane fractions were prepared as described. For detection of vascular ROS, aortic vessel segments (0.4 cm length) were placed in Diogenes™ (ready-to-use) and measured at intervals of 60 s for 20 min and the last value was expressed as counts/min/mg dry weight. Each value was background corrected. For detection of NADPH oxidase generated ROS in membrane fractions the suspensions were diluted to a final protein concentration of 0.2 mg/ml in Diogenes™ (ready-to-use)/PBS 1:4. Upon stimulation with NADPH (200 µM) the chemiluminescence signal was measured at intervals of 30 s for 5 min and the last value was expressed as counts/min. For detection of mitochondrial ROS levels the suspensions were diluted to a final protein concentration of 0.1 mg/ml in Diogenes™ (ready-to-use)/PBS 1:4. Upon stimulation with succinate (2.5 mM) and myxothiazol (10 µM) the chemiluminescence signal was measured at intervals of 30 s for 5 min using a Lumat LB9507 (Berthold Technologies, Bad Wildbad, Germany) and the last value was expressed as counts/min.
Determination of ApoB IgG and IgM Immune Complexes (IgG and IgM IC/apoB)

MAb LF3 (or F_{ab}^{2} fragments of LF3) specific for mouse apoB-100\(^{9}\) was plated on microtiter plates in order to capture mouse apoB-100, and mouse serum (diluted 1:100) was added. In parallel plates, biotin-labeled mAb LF5 (5 µg/ml) was used to determine the relative amounts of mouse apoB-100 bound by LF3. Goat-anti mouse IgG-Fc or IgM (alkaline phosphatase labelled) were used to detect immune complexes to mouse apoB-100 lipoproteins. The relative number of IgG or IgM immune complexes bound per apoB-100 particle was then determined by dividing the bound IgG or IgM relative light units (RLUs, chemiluminescence was read on a MLX microtiter plate luminometer (Dynex Technologies, Chantilly, VA)) by the apoB-100 RLU in parallel wells (IgG-IC/apoB-100, IgM-IC/apoB-100). Each result is the mean of triplicate values per mouse.

Reporter Cell Assay for Determination of Aortic NO Production

Aortic NO production was bioassayed by determination of the cGMP content in RFL-6 rat lung fibroblasts as reporter cells. Thoracic aortae of GPx-1\(^{-/-}\)ApoE\(^{-/-}\) and ApoE\(^{-/-}\) mice after 6 weeks on the WTD were dissected, cleaned of adherent connective tissue, and cut into 2 mm long rings in ice-cold Hank's balanced salt solution (HBSS, Invitrogen, Carlsbad, CA). Confluent RFL-6 cells grown in 12-well plates were washed twice with HBSS and then incubated in HBSS containing 200 U/ml SOD, 10 µM (6R)-5,6,7,8-tetrahydro-L-biopterin (BH\(_{4}\)), 1 mM L-arginine, 0.6 mM 3-isobutyl-1-methylxanthine (IBMX), and two aortic rings in each well for 15 min at 37°C. After preincubation, 300 nM acetylcholine was added and incubation maintained for another 3 min. The reaction was stopped by aspiration of the solution, adding 300 µl of ice-cold 50 mM sodium acetate, pH 4.0, and rapidly freezing the cells with liquid nitrogen. The cGMP content of the RFL-6 samples was determined by a radioimmunoassay as described.\(^{10}\)
Results

Generation of GPx-1<sup>–/–</sup>ApoE<sup>–/–</sup> Mice

PCR whole blood analysis confirmed the presence of mutant alleles for both Gpx-1 (509 bp) and ApoE (245 bp), as well as the absence of their corresponding wild-type alleles (data not shown). Both GPx-1<sup>–/–</sup>ApoE<sup>–/–</sup> and ApoE<sup>–/–</sup> mice were apparently healthy and fertile, and exhibited mendelian transmission of the mutant alleles. We noticed no obvious abnormalities in organ development, gestation time, food/water consumption as well as sleeping and cleaning habit. Compared with ApoE<sup>–/–</sup> mice (309 ± 38.6 U/g Hb), GPx-1<sup>–/–</sup>ApoE<sup>–/–</sup> mice had no detectable red blood cell GPx-1 activity.

Detection of Reactive Oxygen Species Formation in Aortic Vessel Segments, Heart Mitochondria and Membrane Fractions

Basal vascular ROS production (including superoxide, hydrogen peroxide and peroxynitrite) in the aorta was significantly increased in GPx-1<sup>–/–</sup>ApoE<sup>–/–</sup> mice compared to control ApoE<sup>–/–</sup> mice (Figure III, left panel). NADPH-stimulated ROS production in membrane fractions was significantly increased in GPx-1<sup>–/–</sup>ApoE<sup>–/–</sup> mice (Figure III, middle panel). Succinate-stimulated mitochondrial ROS formation showed a significant increase in GPx-1<sup>–/–</sup>ApoE<sup>–/–</sup> mice, which was also present upon blockade of mitochondrial respiration by myxothiazol (Figure III, right panel). Succinate is a substrate of mitochondrial complex II and myxothiazol an uncoupler of complex III. These results point towards increased oxidative stress in GPx-1<sup>–/–</sup>ApoE<sup>–/–</sup> mice due to activation of mitochondria and NADPH oxidases or due to impaired antioxidative defense mechanism in the GPx-1 deficient animals.

Influence of GPx-1 Deficiency on Lipoprotein Oxidation

OxLDL could be detected both extra- and intracellularly in the atherosclerotic lesions of both control and double-knockout mice using antibodies against 4-hydroxynonenal (HNE)-lysine epitopes<sup>11</sup> and oxidized phospholipid epitopes (EO6).<sup>12</sup> The MAb EO6 resulted in a stronger and
more extensive staining than the MAb NA59 but there were no significant differences between control and double-knockout mice at 12 (data not shown) or 24 weeks (Figure IV) on the WTD. IgG-IC/apoB-100, IgM-IC/apoB-100 and IgG and IgM OxLDL autoantibodies were not statistically significant between groups after 12 or 24 weeks on the WTD. However, compared to ApoE−/− mice, double-knockout mice (GPx-1−/−ApoE−/−) showed a trend towards higher titers of IgG-IC/apoB-100 and IgM-IC/apoB-100 as well as IgG autoantibody titers against Cu-OxLDL (data not reaching statistical significance due to high interindividual variations within the groups; not shown).

**Influence of GPx-1 Deficiency on Expression of MCP-1**

To investigate whether increased recruitment of monocytes into the lesions might account for the increased cellularity of early atherosclerotic lesions in GPx-1−/−ApoE−/− mice, we determined MCP-1 protein expression in lesions of mice fed the WTD for 12 weeks. Since MCP-1 protein expression was almost exclusively confined to macrophages, we did not determine percent-positive area for immunohistochemical staining but rather immunostaining intensity. We did not find evidence for an increased staining intensity in macrophages of GPx-1−/−ApoE−/− mice compared to control mice as quantified by Photoshop-based image analysis (supplemental Figure V).

**Vascular NO Production**

To investigate whether endothelial dysfunction might be a mechanism operative during accelerated atherosclerosis in GPx-1−/−ApoE−/− mice, aortic NO production was bioassayed with RFL-6 rat lung fibroblasts as reporter cells. RFL-6 fibroblasts showed basal cGMP levels of approximately 1 pmol/10⁵ cells. In general, incubation of the RFL-6 cells with mouse aortic rings in the presence of 300 nM acetylcholine markedly increased the cGMP content due to acetylcholine-stimulated NO production of the aortic rings. However, aortic rings of GPx-1−/−ApoE−/− mice were significantly less efficient in inducing cGMP production than aortic rings from...
ApoE\textsuperscript{-/-} mice (Figure VI A). These results indicate that less bioactive NO is generated in response to acetylcholine in aortae of double-knockout mice compared to control mice.

**Protein Nitration**

Nitration on the \textit{ortho} position of tyrosine has been demonstrated to be a major product of peroxynitrite attack on proteins.\textsuperscript{13, 14} Using immunohistochemistry, we detected a significant increase in the amount of nitrotyrosine at 12 weeks on the WTD in double-knockout mice compared to control mice (Figure VI B). Immunohistochemical staining was pronounced in and around macrophage-derived foam cells. The observation of extensive protein nitration suggests that peroxynitrite is produced and the significant increase in double-knockout mice suggests that it may be involved in the different evolution of the lesions in control and double-knockout mice.

**References**


**Figure Legends**

**Figure I.** Measurement of plaque size in longitudinal sections of the aortic arch stained with trichrome. A 2-mm segment of the lesser curvature of the aortic arch was defined proximally by a perpendicular axis dropped from the right side of the innominate artery origin (dashed line) and the aortic-arch wall area subtended by this 2-mm stretch of intima (green line) was calculated for each section of all mice by computerized image analysis. The aortic-arch intima thickness (red line) was determined on this same segment of the lesser curvature. IA, innominate artery; LCCA, left common carotid artery; LSA, left subclavian artery.

**Figure II.** GPx-1 deficiency does not affect serum lipids. A, Plasma lipid contents in GPx-1−/− ApoE−/− and ApoE−/− mice at baseline and after 6, 12, and 24 weeks, respectively, on the WTD (mean ± SD). B, Representative lipoprotein profiles after 12 weeks on the WTD. Pooled plasma (n=6) was size-fractionated by FPLC, and the cholesterol content of each fraction was determined by a colorimetric assay.

**Figure III.** Determination of vascular, mitochondrial and NADPH oxidase dependent ROS formation. **Left panel:** Aortic rings (n=3-6) were placed in undiluted Diogenes™ reagent and basal ROS formation was measured over 20 min. **Middle panel:** Heart membrane suspensions (n=5, 0.2 mg/ml protein) in Diogenes™ reagent/PBS 1:4 were counted for 5 min upon
stimulation with NADPH (200 µM). **Right panel:** Cardiac mitochondrial suspensions (n=4-8, 0.1 mg/ml protein) in Diogenes™ reagent/PBS 1:4 were counted for 5 min upon stimulation with succinate (2.5 mM) and myxothiazol (10 µM). Data are expressed as counts/min and presented as boxplots with median, interquartile range, minimum and maximum. * p<0.05 vs. ApoE<sup>-/-</sup> mice, # p<0.05 vs. GPx-1<sup>-/-</sup>ApoE<sup>-/-</sup> mice and $ p<0.05 vs. ApoE<sup>-/-</sup> + myxothiazol group.

**Figure IV.** Influence of GPx-1 deficiency on lipoprotein oxidation. After consuming the WTD for 24 weeks, atherosclerotic lesions of the inner aortic arch intima (lesser curvature) of GPx-1<sup>-/-</sup> ApoE<sup>-/-</sup> and ApoE<sup>-/-</sup> mice were stained with MAb anti-oxidized phospholipid epitopes (EO6, 1:500 (A)), and MAb anti-4-hydroxononenal lysine (NA59, 1:100 (B)). Immunostaining of murine tissues with the murine MAbs was performed using the Vector M.O.M. immunodetection kit. Percent-positive area for immunohistochemical staining (brown color deposits) was quantified by Photoshop-based image analysis. The lumen is to the upper left-hand corner. The demarcation between intima and media is indicated by an arrowhead. Magnification x64.
Data are presented as boxplots with median, interquartile range, minimum and maximum.

**Figure V.** Influence of GPx-1 deficiency on MCP-1 protein expression. After consuming the WTD for 12 weeks, atherosclerotic lesions of the inner aortic arch intima (lesser curvature) of GPx-1<sup>-/-</sup>ApoE<sup>-/-</sup> and ApoE<sup>-/-</sup> mice were stained with rabbit anti-murine JE (MCP-1 (macrophage/monocyte chemotactic protein-1), 1:100). Immunostaining intensity was then quantified by Photoshop-based image analysis and calculated as the difference between MCP-1 immunostaining and background immunostaining (designated immunohistochemical index with arbitrary units (AU)). The lumen is to the upper left-hand corner. The demarcation between intima and media is indicated by an arrowhead. Magnification x64.
Data are presented as boxplots with median, interquartile range, minimum and maximum.
Figure VI. Vascular NO production and protein nitration. A, After 6 weeks on the WTD, acetylcholine stimulated NO production of isolated thoracic aortic rings from GPx-1−/−ApoE−/− and ApoE−/− mice was bioassayed with RFL-6 rat lung fibroblasts as reporter cells. The cGMP content of the RFL-6 samples was determined by radioimmunoassay. B, After consuming the WTD for 12 weeks, atherosclerotic lesions of the inner aortic arch intima (lesser curvature) of GPx-1−/−ApoE−/− and ApoE−/− mice were stained with a polyclonal goat anti-nitrotyrosine antibody using the Vector ABC immunodetection kit. Percent-positive area for immunohistochemical staining (brown color deposits) was quantified by Photoshop-based image analysis. The lumen is to the upper left-hand corner. The demarcation between intima and media is indicated by an arrowhead. Magnification x64.

Data are presented as boxplots with median, interquartile range, minimum and maximum.
**Figure II**

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B

![Diagram showing Cholesterol levels across different time points](image)

- **ApoE-/-**
- **GPx-1-/-ApoE-/-**
Figure III
Figure IV

ApoE-/-
ApoE-/-GPx-1-/-

A

B

ApoE-/-
ApoE-/-GPx-1-/-

NA59
Figure V

ApoE-/-

ApoE-/-GPx-1-/-

immunohistochemical index [AU]

MCP-1

n.s.
Figure VI

(A) Graph showing cGMP (pmol/10^5 RFL-6 cells) production with nitrotyrosine levels. The y-axis represents cGMP levels ranging from 0 to 10, and the x-axis represents NO production. The graph compares ApoE and ApoE-/GPx-1-/- samples, with ApoE-/- samples showing statistically significant differences (p<0.05).

(B) Images showing % positively stained plaque area for nitrotyrosine. The chart indicates ApoE-/- and ApoE-/-GPx-1-/- samples, with ApoE-/- showing significantly lower nitrotyrosine levels compared to ApoE-/-GPx-1-/- (p<0.05).