Lack of the antioxidant glutathione peroxidase-1 does not increase atherosclerosis in C57BL/J6 mice fed a high-fat diet

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Abstract Oxidative stress is thought to contribute to the initiation and progression of atherosclerosis. As glutathione peroxidase-1 (Gpx1) is an antioxidant enzyme that detoxifies lipid hydroperoxides, we tested the impact of Gpx1 deficiency on atherosclerotic processes and antioxidant enzyme expression in mice fed a high-fat diet (HFD). After 12 weeks of HFD, atherosclerotic lesions at the aortic sinus were of similar size in control and Gpx1-deficient mice. However, after 20 weeks of HFD, lesion size increased further in control but not in Gpx1-deficient mice, even though plasma and aortic wall markers of oxidative damage did not differ between groups. In control mice, the expression of Gpx1 increased and that of Gpx3 decreased at the aortic sinus after 20 weeks of HFD, with no change in the expression of Gpx2, Gpx4, catalase, peroxiredoxin-6, glutaredoxin-1 and -2, or thioredoxin-1 and -2. By comparison, in Gpx1-deficient mice, the expression of antioxidant genes was unaltered except for a decrease in glutaredoxin-1 and an increase in glutaredoxin-2. These changes were associated with increased expression of the proinflammatory marker monocyte chemoattractant protein-1 in control mice but not in Gpx1-deficient mice.█ In summary, a specific deficiency in Gpx1 was not accompanied by an increase in markers of oxidative damage or increased atherosclerosis in a murine model of HFD-induced atherogenesis.—de Haan, J. B., P. K. Witting, N. Stefanovic, J. Pete, M. Daskalakis, I. Kola, R. Stocker, and J. J. Smolich. Lack of the antioxidant glutathione peroxidase-1 does not increase atherosclerosis in C57BL/J6 mice fed a high-fat diet. *J. Lipid Res.* 2006. **47:** 1157–1167.

Supplementary key words antioxidant enzymes • lipid hydroperoxides • knockout mice • oxidative stress

The likelihood and extent of atherosclerosis, a major cause of morbidity and mortality in Western societies, is affected by a number of known risk factors, including hypercholesterolemia (1), hypertension (2), diabetes (2), hyperhomocysteinemia (3), and inflammation (4). In addition, increased oxidative stress within the vasculature, manifested by the accumulation of oxidized lipids and proteins as well as the formation of reactive oxygen species, is commonly considered to play a key role in the initiation and progression of atherogenesis (5–7). To regulate the flux of reactive oxygen species and to limit oxidative damage, eukaryotic cells have evolved an extensive array of antioxidant defense systems, including intracellular antioxidant enzymes (8, 9). Recent evidence from mice deficient in apolipoprotein E, a widely used model of atherosclerosis, suggests that the levels of several such enzymes decline during atherogenesis (10), implying a link between reduced antioxidant capacity and increased disease. However, limited information is available on the involvement of individual antioxidant enzymes in atherogenic processes.

Glutathione peroxidase-1 (Gpx1) is a major and ubiquitous antioxidant enzyme present in the cytosol and mitochondria and involved in the detoxification of H_2O_2 and lipid hydroperoxides (11). In the absence of Gpx1, a buildup of H_2O_2 has the potential to damage DNA, proteins, and lipids (12–14). Several lines of evidence indirectly suggest a potential role for Gpx1 in atherogenesis. Thus, a recent prospective cohort study showed that reduced red blood cell Gpx1 activity is a strong predictor of cardiovascular events (15), and Gpx1 activity is reduced in

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atherosclerotic plaques of patients with carotid artery disease (16). In addition, patients with selenium deficiency display an increased incidence of atherosclerotic events (17) that was attributed to decreased Gpx activity, because selenium is an essential cofactor for Gpx function (11). Furthermore, hyperhomocysteinemia is associated with a significant reduction in Gpx-1 expression and increased risk of cardiovascular disease (18, 19). To date, only a few studies have directly examined the consequences of reduced Gpx1 on the vasculature. One study showed structural abnormalities, including intimal thickening in aged mice with a heterozygous deficiency in Gpx1 (20). Another study reported that Gpx1 deficiency sensitized hyperhomocysteinemic mice to endothelial dysfunction (21). However, no previous study has directly tested the effect of decreased Gpx1 activity on atherogenesis.

Accordingly, the aim of this study was to determine plasma and aortic wall lipid concentrations, as well as enzymatic and nonenzymatic antioxidant profiles, in Gpx1-deficient mice (22) fed a high-fat diet (HFD) and to relate these parameters to atherosclerotic lesion size in the aortic sinus, a region known to be highly susceptible to atherogenesis in the mouse (23). Our results show that the HFD did not significantly alter plasma and aortic lipid or antioxidant profiles in Gpx1-deficient mice (with the exception of glutaredoxin-1 and -2); nor, compared with control mice, did it increase the extent of atherosclerosis at this site. Rather, after long-term HFD, the extent of atherosclerosis and the expression of the proinflammatory marker monocyte chemotactic peptide-1 were less pronounced in Gpx1-deficient mice than in control mice. Our findings imply that the absence of functional Gpx1 does not enhance the susceptibility of mice to HFD-induced atherosclerosis.

MATERIALS AND METHODS

Animal groups and experimental design

Mice lacking functional Gpx1 were generated via homologous recombination on a mixed genetic background, as described previously (22). Because susceptibility to atherosclerosis in mice is known to be greater in certain background strains (24), Gpx1-deficient mice of mixed background were then bred onto the highly susceptible C57BL/J6 genetic background (nine backcrosses). Standard C57BL/J6 control mice were generated through the breeding program and maintained as a separate line. All studies were performed in pure genetic background, female, age-matched control or Gpx1-deficient mice, because male mice are more resistant to atherosclerosis (23).

After weaning, mice were housed in groups of five and fed normal rodent diet (19% protein, 4.5% fat, 4.5% crude fiber with added vitamins [0.01% DL-α-tocopherol (α-TOH), 10,000 IU/kg vitamin A, 0.003% vitamin B_{12}, 2,000 IU/kg vitamin D_3, with no added ascorbate] and minerals (0.77% calcium, 0.57% phosphorus, and 0.57% salt; GlenForrest Stock Feeders) until 8 weeks of age. Thereafter, animals were either maintained on the normal diet (ND) or fed a HFD ad libitum according to Nishina et al. (25), while having free access to water. The HFD was manufactured commercially (GlenForrest Stock Feeders) and contained 1% cholesterol, 15% fat (cocoa butter), 50% sucrose, 20% casein, 1% canola oil, 5.07% cellulose, 1% AIN-93G vitamins

(0.01% DL-α-TOH, 4,000 IU/kg vitamin A, 0.01% vitamin B_{12}, 1,000 IU/kg vitamin D_3, with no added ascorbate), 1% choline chloride, 0.3% DL-methionine, and 0.5% sodium cholate.

All studies were performed with appropriate approval of the Institutional Ethics Committee. Initial experiments in control and Gpx1-deficient mice were performed after 12 (12 wk-HFD) or 20 (20 wk-HFD) weeks of HFD, with analysis of plasma and aortic wall biochemistry as well as lesion area within the aortic sinus region. All animals tolerated the HFD, with similar weight gain compared with animals receiving the ND. As preliminary observations indicated that measured parameters were similar at the two time points examined in mice fed the ND, the control group consisted of 20 weeks of ND (20 wk-ND). Based on the results of the initial experiments, additional studies were performed to examine the expression of a range of antioxidant enzymes and markers of inflammation in the aortic sinus region after 20 wk-ND or 20 wk-HFD in control and Gpx1-deficient mice, using quantitative reverse transcription-polymerase chain reaction.

Blood sampling and plasma biochemistry

Mice were anesthetized by intraperitoneal injection of 2.5% (0.5 ml/20 g mouse) 2,2,2-tribromoethanol (Avertin in saline; Sigma Chemical Co.) after food had been withheld for 3–4 h but free access provided to water. The thoracic cavity was rapidly opened and 0.5–0.8 ml of blood was drawn into a 1 ml syringe by direct puncture of the right ventricle. Blood samples were placed into heparinized tubes, and plasma was obtained according to Letters et al. (26). Aliquots of plasma were then frozen at −80°C for later analysis of lipid and the nonenzymatic antioxidants α-TOH and total coenzyme Q (CoQ) (27). Other aliquots were diluted in 5% metaphosphoric acid before storage at −80°C and subsequent analysis for the nonenzymatic antioxidants ascorbate (vitamin C) and urate (26). Reverse-phase HPLC was used to measure α-TOH and CoQ as well as hydroperoxides and hydroxides of cholesteryl esters [CE-O(O)H]. Cholesterol, HDL, and triacylglycerols were measured with standard commercial enzymatic kits using the Dimension RxL Chemistry Analyser (Dade Behring Diagnostics, Sydney, Australia). The Friedewald calculation was used to determine levels of LDL.

After blood sampling, mice were either perfused with a buffer solution for determination of aortic wall parameters or fixed by perfusion for microscopic examination and morphometric analysis of the aortic sinus region (26).

Aortic wall biochemistry

Procedures were essentially as described previously (26). Each study group contained 16 mice in which the entire aortic tree was perfused for 5 min at a hydrostatic pressure of 80 cm H_2O with Dulbecco's phosphate-buffered saline containing 5 μM butylated hydroxytoluene and 2 mM EDTA [buffer A, prepared according to Letters et al. (26)]. Perfusion was carried out via a cannula introduced into the left ventricle, with incision of the right atrial appendage to permit outflow of blood and perfusate. At the end of the perfusion procedure, hearts with the thoracic and abdominal aortas attached were excised and placed into buffer A and cleaned of extraneous fat using a dissecting microscope. Because of the small amount of material obtained from each mouse, four aortas were pooled for biochemical analyses. Pooled aortas were frozen in buffer A and stored at −80°C until analyses were performed. At that time, the pooled aortas were pulverized in liquid N_2 and resuspended in buffer A. A 50 μl aliquot was removed for protein determination using the bicinchoninic acid assay kit (Sigma) with BSA as standard, and the remainder was extracted in 500 μl aliquots. This fraction was analyzed by reverse-phase HPLC (27) for α-TOH, CoQ, cholesterol, cholesteryl

arachidonate (C20:4), and cholesteryl linoleate (C18:2). Analysis was also undertaken for CE-O(O)H to determine the extent of aortic lipoprotein lipid oxidation. All biochemical parameters were normalized against total aortic protein.

Morphometric analysis of the aortic sinus region

Each study group contained seven to nine mice. These were perfused at a pressure of 80 cm H_2O with phosphate-buffered saline (pH 7.0) for 5 min followed by 4% paraformaldehyde in phosphate-buffered saline for 5 min via a cannula introduced into the left ventricle, with incision of the right atrial appendage to permit outflow of blood and perfusate. After removal of the heart with the ascending aorta attached, the heart was cut at a plane parallel to the atrial appendages, washed three times in 30% sucrose (15 h duration each), and embedded in OCT compound (Tissue-Tek; Sakura Finetechnical Co., Tokyo, Japan). Frozen cryostat sections were cut at 10 μm intervals from the left ventricular outflow tract through the aortic sinus, according to Paigen et al. (23). Beginning where the aortic leaflets first become visible, four sections were selected at 80 μm intervals from the aortic sinus region (i.e., sampling covered a distance of 320 μm), stained with Oil Red O to delineate lipid deposits, and counterstained with hematoxylin. The aortic sinus region was evaluated because this portion of the aorta is particularly susceptible to the development of atherosclerotic lesions in mice fed HFD (23). Sections of the aortic sinus region were examined using light microscopy at ×40 magnification with an Olympus BX60 optical microscope (Olympus Optical Co., Hamburg, Germany), and the cross-sectional area of lipid depositions was quantified using image-analysis software (Image ProPlus-4; Scitech). For each mouse, the lesion size was measured in three to four cross-sections, and the lesion size per cross-section was averaged to provide the mean lesion size per mouse.

Expression of antioxidant enzymes and proinflammatory markers

After 20 weeks of ND or HFD, control and Gpx1-deficient mice were anesthetized by intraperitoneal injection of 2.5% Avertin in saline, after food had been withheld for 3–4 h but free access provided to water. The thoracic cavity was rapidly opened, and hearts were excised and immediately placed in cold saline. After careful dissection of the aortic sinus region with a dissecting microscope, the tissue was snap-frozen in liquid nitrogen and stored at −70°C until required. Total RNA was extracted after homogenization of tissue (Polytron PT-MR2100; Kinematica AG) in TRIzol® reagent (Invitrogen Life Technologies). Contaminating DNA was removed after treatment with DNA-free™ DNase according to the manufacturer's specifications (Ambion Inc., Austin, TX). Finally, DNA-free RNA was reverse-transcribed into cDNA using the Superscript First Strand Synthesis System according to the manufacturer's specifications (Life Technologies BRL, Grand Island, NY). The expression of the genes coding for the antioxidant enzymes Gpx1, Gpx2, Gpx3, Gpx4, catalase, peroxiredoxin-6, glutaredoxin-1, glutaredoxin-2, thioredoxin-1, and thioredoxin-2 as well as the proinflammatory markers monocyte chemoattractant protein-1 (MCP-1) and vascular cell adhesion molecule-1 (VCAM-1) was analyzed by quantitative RT-PCR using the Taqman system based on real-time detection of accumulated fluorescence (ABI Prism 7700; Perkin-Elmer, Inc., Foster City, CA). Fluorescence for each cycle was analyzed quantitatively, and gene expression was normalized relative to the expression of the housekeeping gene 18S rRNA (18S rRNA Taqman Control Reagent kit) multiplexed together with the gene of interest. Probes and primers were designed using the Primer Express program with care taken to ensure that primers

spanned an intron (**Table 1**). Probes and primers were purchased from Applied Biosystems (Foster City, CA). Amplifications were performed with the following time course: 50°C for 2 min, 95°C for 10 min, and 40 cycles of 94°C for 20 s and 60°C for 1 min. Samples were tested in duplicate, and results are expressed relative to wild-type mice fed ND, which were arbitrarily assigned a value of 1.

Statistical analysis

Plasma and aortic wall biochemical, aortic sinus morphometric, and aortic sinus quantitative reverse transcription-polymerase chain reaction data were analyzed using one-way ANOVA and a posthoc Tukey's multiple comparison test (GraphPad Prism 4.0; GraphPad Software, Inc., San Diego, CA). Results are expressed as means ± SEM, and $P < 0.05$ was considered statistically significant.

RESULTS

Plasma biochemistry

Plasma biochemical data are presented in **Table 2**. Lipids and antioxidants were similar in 20 wk-ND control and Gpx1-deficient mice. However, the levels of nonesterified cholesterol were increased ∼2-fold after 12 and 20 weeks of HFD in both control and Gpx1-deficient mice, suggesting that a similar degree of lipid loading was achieved in both groups.

In control mice, the concentration of triacylglycerols was unchanged after 12 weeks of HFD and increased after 20 weeks of HFD ($P < 0.01$) but was unaltered in Gpx1-deficient mice at either time point. As a result, the triacylglycerol level was less in Gpx1-deficient mice than in control mice ($P < 0.001$) after 20 weeks of HFD. Compared with control animals, HDL was higher in Gpx1-deficient mice fed HFD for 20 weeks ($P < 0.01$), whereas LDL was higher in all HFD-fed than ND-fed groups ($P < 0.001$), resulting in an ∼6-fold greater LDL/HDL ratio in HFD-fed mice ($P < 0.01$).

Compared with ND mice, levels of α-TOH and ascorbate were increased ∼5-fold in HFD control and Gpx1-deficient animals ($P < 0.001$), without any difference between control and Gpx1-deficient animals after 12 or 20 weeks of HFD. The observed increase in plasma α-TOH after HFD feeding is similar to that reported in apolipoprotein E-deficient mice fed a Western diet (26) and is suggestive of greater absorption of the lipid-soluble vitamin with HFD, as ND and HFD contained similar amounts of DL-α-TOH. Importantly, the concentrations of α-TOH, ascorbate, CoQ, and urate were similar in all HFD groups, excluding the possibility that differences in these antioxidants influenced disease outcomes. Moreover, total CoQ and urate were not significantly different in the control and Gpx1-deficient mice receiving ND, whereas CE-O(O)H was not significantly different between groups.

Aortic wall biochemistry

Aortic wall biochemical data are presented in **Table 3**. Aortic lipids and antioxidants were not significantly different in control and Gpx1-deficient ND mice. The HFD

TABLE 1. Primers and probes used for quantitative RT-PCR

Gpx1	Forward primer	CCC CAC TGC GCT CAT GA
	Reverse primer	GGC ACA CCG GAG ACC AAA
	Probe	6FAM CGA CCC CAA GTA CAT C MGBNFQ
Gpx2	Forward primer	TGA CCC GTT CTC CCT CAT
	Reverse primer	GCG CAC GGG ACT CCA TAT
	Probe	6FAM CGA TCC CAA GCT CAT MGBNFQ
Gpx3	Forward primer	GCC AGC TAC TGA GGT CTG ACA GA
	Reverse primer	CAA ATG GCC CAA GTT CTT CTT G
	Probe	6FAM TAC CTT GAA CTG AAT GCA CT MGBNFQ
Gpx4	Forward primer	GCT GTG CGC GCT CCA T
	Reverse primer	CCA TGT GCC CGT CGA TGT
	Probe	6FAM CAC GAA TTC TCA GCC AAG MGBNFQ
Catalase	Forward primer	TTC AGA AGA AAG CGG TCA AGA AT
	Reverse primer	GAT GCG GGC CCC ATA GTC
	Probe	6FAM CAC TGA CGT CCA CCC MGBNFQ
Peroxiredoxin-6	Forward primer	CCT GAA GAG GAA GCC AAA CAA
	Reverse primer	CGG AGG TAT TTC TTG CCA GAT
	Probe	6FAM TCC CTA AAG GAG TCT TCA C MGBNFQ
Thioredoxin-1	Forward primer	TGC AGA GGG CCA AAG TTC A
	Reverse primer	TGG AAC TGG AGG AAC AAG TAG CT
	Probe	6FAM TTC TCA GCA TCC ATA CGG MGBNFQ
Thioredoxin-2	Forward primer	CAG CCT CTG GCA CAT TTC CT
	Reverse primer	GTT CGG CTT CTG GTT TCC TTT
	Probe	6FAM CCT GCC TCT GCT TGA MGBNFQ
Glutaredoxin-1	Forward primer	CCC TTC CCA CTC CTG CAT T
	Reverse primer	GGA GGT TGA GGC TGA GAA CAC T
	Probe	6FAM ACT GCC CTT ACT TAG C MGBNFQ
Glutaredoxin-2	Forward primer	TTT GTC AAT GGA CGA TTT ATT GGA
	Reverse primer	GCA GCA ATT TCC CTT CTT TGT G
	Probe	6FAM CGC ACG GAC ACT C MGBNFQ
Vascular cell adhesion molecule-1	Forward primer	CTG CTC AAG TGA TGG GAT ACC A
	Reverse primer	ATC GTC CCT TTT TGT AGA CAT GAA G
	Probe	6FAM CCA AAA TCC TGT GGA GCA G MGBNFQ
Monocyte chemoattractant protein-1	Forward primer	GTC TGT GCT GAC CCC AAG AAG
	Reverse primer	TGG TTC GAT CCA GGT TTT TA
	Probe	6FAM AAT GGG TCC AGA CAT AC MGBNFQ
18S	Forward primer	TGT TCA CCA TGA GGC TGA GAT C
	Reverse primer	TGG TTG CCT GGG AAA ATC C
	Probe	VIC TGC TGG CAC CAG ACC AGA CTT GCC CTCTAMRA

Gpx, glutathione peroxidase.

had no significant effect on cholesterol levels, nor did it affect C18:2, C20:4, or total CE in control or Gpx1-deficient mice. In Gpx1-deficient mice, aortic wall α-TOH was higher in 20 wk-HFD mice than in 20 wk-ND mice ($P < 0.05$), a finding most likely attributable to the 10-fold lower level of α-TOH in ND Gpx1-deficient aortas. However, importantly, no significant difference in α-TOH was noted between control and Gpx1-deficient groups after 20

TABLE 2. Plasma lipid and antioxidant profiles in C57BL/J6 control and Gpx1-deficient mice fed ND or HFD for 12 or 20 weeks

Parameter	20 wk-ND		12 wk-HFD		20 wk-HFD	
	Control	Gpx1-Deficient	Control	Gpx1-Deficient	Control	Gpx1-Deficient
Cholesterol	1.6 ± 0.1	1.6 ± 0.1	3.3 ± 0.3[a]	3.4 ± 0.28[b]	3.8 ± 0.3[c]	4.9 ± 0.6[c]
Triacylglycerol	0.55 ± 0.04	0.61 ± 0.05	0.37 ± 0.02	0.29 ± 0.02	1.23 ± 0.3[b]	0.4 ± 0.05[d]
HDL	1.1 ± 0.05	1.0 ± 0.05	1.1 ± 0.15	1.2 ± 0.15	1.0 ± 0.01	1.9 ± 0.3[b]
Calculated LDL	0.2 ± 0.03	0.2 ± 0.03	1.6 ± 0.1[c]	1.6 ± 0.05[c]	1.5 ± 0.08[c]	1.8 ± 0.15[c]
LDL/HDL ratio	0.2 ± 0.05	0.2 ± 0.03	1.3 ± 0.15[c]	1.3 ± 0.1[c]	1.4 ± 0.05[c]	1.0 ± 0.2[b]
α-TOH	3.9 ± 0.3	4.6 ± 0.4	21.7 ± 1.8[c]	19.9 ± 1.4[c]	23.7 ± 1.6[c]	20.8 ± 0.9[c]
Total CoQ	1.10 ± 0.4	0.71 ± 0.3	0.63 ± 0.3	0.52 ± 0.15	1.39 ± 0.3	1.18 ± 0.2
Ascorbate	29 ± 7.3	28 ± 8.8	155 ± 14.3[c]	134 ± 11.3[c]	141 ± 8.3[c]	126 ± 20.3[c]
Urate	87 ± 21.8	72 ± 7.8	58 ± 3.5	67 ± 4.8	60 ± 5.5	83 ± 10.3
CE-O(O)H	0.03 ± 0.03	0.09 ± 0.05	0.16 ± 0.05	0.19 ± 0.05	0.13 ± 0.03	0.18 ± 0.05

CoQ, coenzyme Q; HFD, high-fat diet; ND, normal diet; α-TOH, α-tocopherol; 12 wk-HFD, 12 weeks of HFD; 20 wk-HFD, 20 weeks of HFD; 20 wk-ND, 20 weeks of ND. Data represent means ± SEM of 12–16 plasma samples. Values for antioxidants and CE-O(O)H are expressed in μmol/l. Cholesterol, triacylglycerol, HDL, and calculated LDL are expressed in mmol/l. Total CoQ represents the sum of CoQ_9 and CoQ_{10}. CE-O(O)H represents both hydroperoxides and hydroxides of cholesteryl esters.
[a] $P < 0.05$ compared with the corresponding ND value.
[b] $P < 0.01$ compared with the corresponding ND value.
[c] $P < 0.001$ compared with the corresponding ND value.
[d] $P < 0.001$ compared with the 20 wk-HFD control value.

TABLE 3. Aortic wall lipid and antioxidant profiles in C57BL/J6 control and Gpx1-deficient mice fed ND or HFD for 12 or 20 weeks

Parameter	20 wk-ND		12 wk-HFD		20 wk-HFD	
	Control	Gpx1-Deficient	Control	Gpx1-Deficient	Control	Gpx1-Deficient
Cholesterol	61 ± 15	31 ± 11	51 ± 11	55 ± 15	56 ± 21.5	76 ± 15
C20:4	0.02 ± 0.01	0.01 ± 0.01	0.02 ± 0.01	0.05 ± 0.02	0.05 ± 0.03	0.06 ± 0.01
C18:2	6.4 ± 2.2	1.9 ± 0.8	7.1 ± 1.3	5.8 ± 3.0	4.9 ± 2.2	4.5 ± 0.8
Total cholesteryl ester	6.4 ± 2.2	1.9 ± 0.8	7.1 ± 2.0	5.9 ± 3.0	4.9 ± 2.2	4.5 ± 0.8
α-TOH	1.1 ± 0.1	0.1 ± 0.1	3.0 ± 0.7	3.2 ± 1.0	3.0 ± 1.2	4.3 ± 0.9[a]
α-TOH/cholesterol	0.02 ± 0.02	0.01 ± 0.01	0.07 ± 0.03	0.06 ± 0.03	0.05 ± 0.03	0.05 ± 0.03
Total CoQ	0.16 ± 0.08	0.06 ± 0.03	0.10 ± 0.03	0.12 ± 0.06	0.09 ± 0.04	0.09 ± 0.03
CE-O(O)H	0.17 ± 0.1	0.06 ± 0.03	0.11 ± 0.03	0.08 ± 0.03	0.09 ± 0.04	0.1 ± 0.03
CE-O(O)H/cholesteryl ester	0.03 ± 0.01	0.03 ± 0.01	0.02 ± 0.002	0.02 ± 0.01	0.02 ± 0.01	0.02 ± 0.01

C18:2, cholesteryl linoleate; C20:4, cholesteryl arachidonate. Data are expressed as means ± SEM of four pooled samples. Each pooled sample consisted of four aortas. Values for cholesterol, cholesteryl ester, antioxidants, and lipid peroxidation products are expressed as nmol/mg protein. Total CoQ represents the sum of oxidized and reduced CoQ_{10}. CE-O(O)H represents cholesteryl ester-derived lipid hydroperoxides and hydroxides. Total CE represents the sum of C18:2 and C20:4.
[a] $P < 0.05$ compared with the corresponding ND value.

weeks of HFD, eliminating the likelihood that increased α-TOH levels in Gpx1-deficient aortas affected atherosclerosis. Furthermore, the α-TOH/cholesterol ratio was unchanged in any of the groups studied. Similarly, total CoQ levels were not significantly different between groups.

Cholesteryl ester-derived lipid hydroperoxides plus hydroxides were detected in the aortic wall of all groups but without any significant difference between groups, independent of whether results were normalized against total protein or cholesteryl esters.

Aortic sinus morphometry

Lipid accumulation was not apparent in the aortic sinus region of control and Gpx1-deficient mice receiving 20 weeks of ND (data not shown). By contrast, Oil Red O-positive foam cells were evident in the intimal space between the intact endothelial cell layer and the subjacent smooth muscle cell layer in both HFD control and Gpx1-deficient mice (**Fig. 1**). However, the area of lipid deposition was not significantly different in control and Gpx1-deficient mice after 12 weeks of HFD (Fig. 1A, C, **Fig. 2**). In control mice, the area of lipid deposition increased 2.6-fold between 12 and 20 weeks of HFD ($P < 0.001$) (Figs. 1A, B, 2). By contrast, in Gpx1-deficient mice, the area of lipid deposition after 20 weeks of HFD was not significantly different from that measured after 12 weeks of HFD ($P > 0.5$) (Figs. 1C, D, 2). As a result, the area of lipid deposition after 20 weeks of HFD was significantly greater in control mice than in Gpx1-deficient mice ($P < 0.001$) (Figs. 1B, D, 2).

Aortic sinus antioxidant enzymes and proinflammatory markers

The aortic content of Gpx1 mRNA increased by ∼20-fold ($P < 0.001$) in control mice after 20 weeks of HFD (**Fig. 3A**), perhaps reflecting a compensatory response to increased oxidative stress proposed to be associated with the consumption of HFD (28). However, and as expected of a model in which homologous recombination has disrupted gene function (22), Gpx1 expression remained unchanged in 20 wk-HFD Gpx1-deficient mice (Fig. 3A). Interestingly, in aortic tissue from control mice, 20 weeks

of HFD did not affect the expression of Gpx2 (Fig. 3B) or Gpx4 (Fig. 3D) mRNA, whereas it reduced Gpx3 mRNA by 40% ($P < 0.05$) (Fig. 3C). In Gpx1-deficient mice, Gpx2 mRNA was increased after 20 weeks of ND, but this was not evident after 20 weeks of HFD (Fig. 3B).

Assessment of genes for other antioxidant enzymes revealed no significant changes in the expression of catalase (**Fig. 4A**), peroxiredoxin-6 (Fig. 4B), glutaredoxin-1
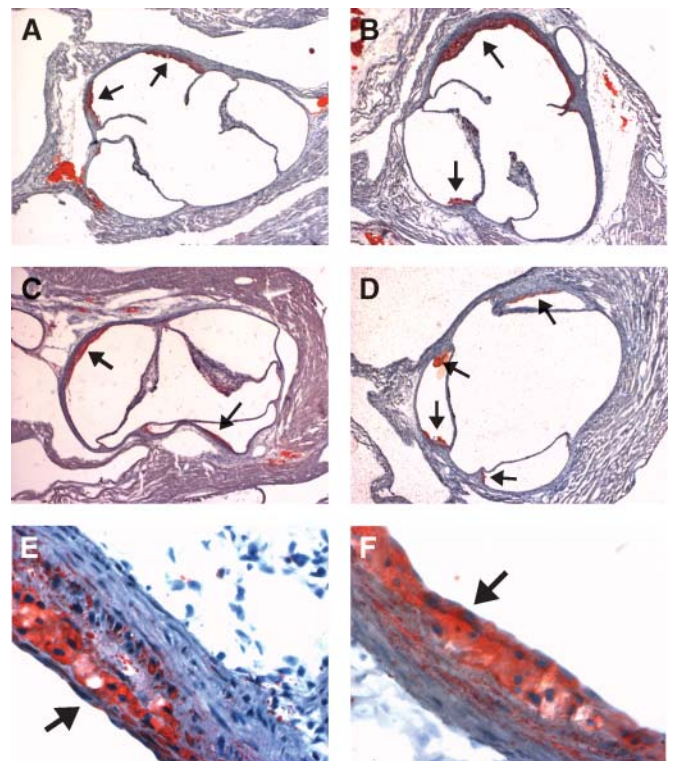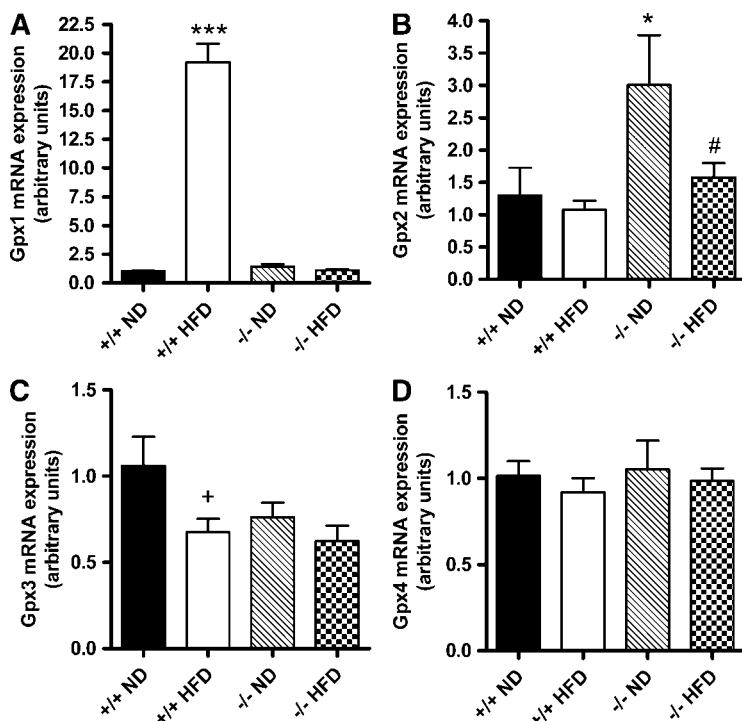

Fig. 1. Oil Red O staining of atherosclerotic lesions within the aortic sinus region of control and glutathione peroxidase-1 (Gpx1)-deficient mice fed a high-fat diet (HFD). A: Control aorta after 12 weeks of HFD. B, E: Control aortas after 20 weeks of HFD. C: Gpx1-deficient aorta after 12 weeks of HFD. D, F: Gpx1-deficient aortas after 20 weeks of HFD. Magnification: ×40 (A–D) and ×400 (E, F). Arrows in A–D indicate lesions within the aortic wall; arrows in E, F point to the intact single-cell endothelial layer of the arterial wall.
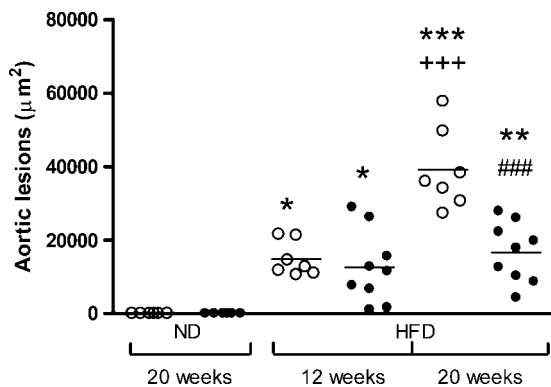
Fig. 2. Atherosclerotic lesion area (μm^2) in the aortic sinus region of control (open circles) and Gpx1-deficient (closed circles) mice after 20 weeks of normal diet (ND) and 12 and 20 weeks of high fat diet (HFD). Each symbol represents the area of the sinus stained for lipid with Oil Red O in a single mouse. No lesions were detected after 20 weeks of ND in either group. All groups fed HFD developed significant lesions after 12 weeks (* $P < 0.05$) and 20 weeks (*** $P < 0.001$, ** $P < 0.01$) compared with ND counterparts. Lesions detected in aortas of control mice on 20 weeks of HFD were significantly increased compared with those of control mice on 12 weeks of HFD ($^{+++}$ $P < 0.001$). Lesions detected in aortas of 20 wk-HFD Gpx1-deficient mice were significantly reduced compared with those in 20 wk-HFD controls ($^{\#\#\#}$ $P < 0.001$). Horizontal bars represent mean values for each group.

(**Fig. 5A**), thioredoxin-1 (Fig. 5C), or thioredoxin-2 (Fig. 5D) in Gpx1-deficient versus control mice receiving HFD. However, glutaredoxin-1 mRNA was decreased in Gpx1-deficient mice receiving HFD rather than ND for 20 weeks (Fig. 5A), whereas glutaredoxin-2 mRNA was increased ~2-fold in Gpx1-deficient mice after 20 weeks of HFD compared with control animals fed ND or HFD (Fig. 5B).

Evaluation of inflammatory markers that are thought to contribute to atherogenesis (29), indicated that expression of MCP-1 increased by ~50% after 20 weeks of HFD in the aortic sinus of control mice ($P < 0.05$) but was unaffected by the deficiency of Gpx1 with either ND or HFD (**Fig. 6A**). Expression of VCAM-1 was unaffected by HFD in control and Gpx1-deficient mice but was increased by ~50% ($P < 0.05$) in Gpx1-deficient animals after 20 weeks of ND (Fig. 6B).

DISCUSSION

In this study, we used a targeted approach to address the question of whether a specific elimination of Gpx1 function in a murine model of atherosclerosis leads to increased lesion formation at the aortic root, a site known to be predisposed to atherogenesis. We based our rationale on the notion that a deficiency in Gpx1 results in diminished antioxidant defense, such that oxidative stress increases, and that this enhanced oxidative stress may translate into accelerated disease progression. Specifically, a deficiency in the antioxidant enzyme Gpx1 would be expected to increase intracellular concentrations of H_2O_2 and fatty acid hydroperoxides, both of which could adversely affect vascular cell function (30, 31). Furthermore, oxidative stress is expected to increase after consumption of HFD per se, with subsequent upregulation of a proinflammatory state that itself promotes atherogenesis (28). Indeed, this study lends support to the notion that oxidative stress is increased by HFD, because we showed previously that Gpx1 expression and activity are enhanced by reactive oxygen species (22), and we now show that Gpx1 is the only Gpx isoform and the only
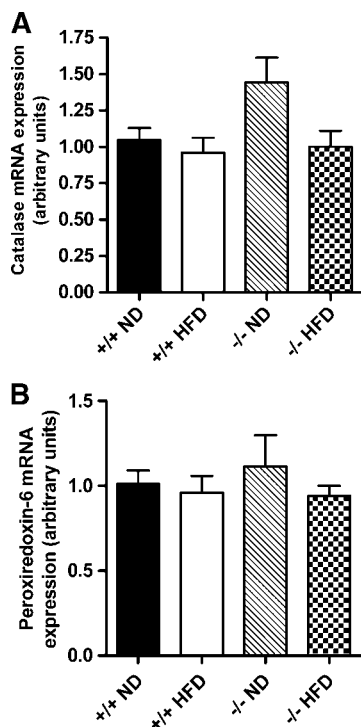

Fig. 3. Gene expression of Gpx isoforms in the aortic sinus region of control (+/+) and Gpx1-deficient (−/−) mice after 20 weeks of either ND or HFD. A: Gpx1. B: Gpx2. C: Gpx3. D: Gpx4. For each gene investigated, gene expression is expressed relative to the control group fed ND, which is arbitrarily designated as 1. n = 5 for ND groups; n = 10 for HFD groups. Bars represent means ± SEM. *** $P < 0.001$ versus +/+ ND group; * $P < 0.05$ versus +/+ ND group; $^{\#}$ $P < 0.05$ versus −/− ND group; $^{+}$ $P < 0.05$ versus +/+ ND group.

Fig. 4. Gene expression of catalase (A) and peroxiredoxin-6 (B) in the aortic sinus region of control (+/+) and Gpx1-deficient (−/−) mice after 20 weeks of ND or HFD. For each gene investigated, gene expression is expressed relative to that in the control group fed ND, which is arbitrarily designated as 1. n = 5 for ND groups; n = 10 for HFD groups. Bars represent means ± SEM. No significant differences in expression were noted for any of the groups investigated.

enzymatic antioxidant, at the expression level at least, to show a dramatic increase in mice fed a HDF. Oxidant stress, therefore, is expected to be greatly increased in Gpx1-deficient aortas that are unable to respond to this increased stress and, based on previous supportive literature (5–7), could be expected to contribute to atherogenic processes. Thus, our observation that lesion size in the aortic sinus was not different after 12 weeks of HFD and decreased after 20 weeks of HFD in mice lacking Gpx1, compared with control animals, was unexpected. Furthermore, we observed that GPx1 deficiency was accompanied by unchanged aortic concentrations of oxidized lipoprotein lipids as well as an unaltered profile of the major antioxidants involved in the cellular peroxide balance. Moreover, expression of the proinflammatory markers MCP-1 and VCAM-1 was unaffected in Gpx1-deficient mice fed HFD. Our findings suggest that the link between reduced Gpx1 function and a predisposition to atherosclerosis and inflammation, if existent, is more complex than suggested previously using indirect approaches (15, 16, 32, 33). Furthermore, our data in 20 week HFD-fed Gpx1-deficient mice is suggestive of protective mechanism(s) that are activated by the lack of Gpx1 and, by inference, that hydrogen and/or lipid peroxides may be involved in this protection.

The results of our study are not immediately consistent with previous in vitro studies investigating the effects of reduced Gpx1 activity on putative proatherogenic processes. One study, examining the effect of Gpx status on the ability of macrophages to oxidize LDL, observed that decreasing Gpx activity via inhibition of glutathione synthesis was accompanied by a 2-fold increase in LDL oxidation (32). Another study observed LDL oxidation and oxidized LDL-induced apoptosis to be significantly increased when native LDL was incubated with aortic segments and smooth muscle cells obtained from Gpx1-deficient compared with control mice (33). Importantly however, although these studies concluded that decreased Gpx1 activity may accelerate atherogenesis, they did not examine atherogenic processes, in contrast to our studies reported here, which examined the effects of an absence of Gpx1 on aortic lesion formation.

In contrast to the in vitro studies described above, our results are more consistent with previous studies using in vivo models. Thus, Wang et al. (34) reported recently that atherosclerosis was not affected by the ablation of peroxiredoxin-6 in mice. Peroxiredoxin is a member of the newly identified family of antioxidant enzymes that act on H_2O_2 and alkyl hydroperoxides (35). Similarly, Tribble et al. (36) reported no difference in atherosclerotic lesions in control C57BL/6J mice compared with animals overexpressing Cu,Zn-superoxide dismutase (Sod1). However, when these mice were exposed to ionizing radiation, Sod1 transgenic mice developed smaller lesions than control animals (37). Interestingly, the increased removal of superoxide anion radicals resulting from the overexpression of Sod1 is expected to increase cellular H_2O_2 (38). Thus, in both our model (22) and the Sod1 transgenic model (37), increased cellular H_2O_2 is associated with decreased atherosclerosis in C57BL/6J mice fed a HFD. Whether this extends to other models of atherosclerosis remains to be tested. One study, however, has shown that overexpression of catalase reduced the severity of lesions in apolipoprotein E-deficient mice (39), implying that H_2O_2 contributes to atherosclerosis in this model.

A number of mechanisms may have contributed to the lack of progression of atherosclerotic lesions in Gpx1-deficient mice in our study. First, known antiatherogenic factors may have limited atherogenesis after prolonged HFD feeding. In particular, compared with controls, we found increases in HDL and reduced triacylglycerol levels in Gpx1-deficient mice after 20 weeks of HFD. Calorie restriction has been reported to reduce plasma triacylglycerols, increase HDL, and retard the development of atherosclerosis (40). However, we can exclude this possibility, because our animals received diet ad libitum and both groups of mice achieved similar weight gain over the period of study. Furthermore, HDL has several atheroprotective activities (41), so that the observed increase in HDL concentration in Gpx1-deficient mice may explain the absence of increased atherosclerosis. However, it is important to note in this context that we measured lipoproteins only in the blood, and it is not known how precisely this reflects their respective levels in the aortic wall, where atherosclerotic lesions were assessed. Furthermore, the link between HDL and Gpx1 deficiency remains unclear.
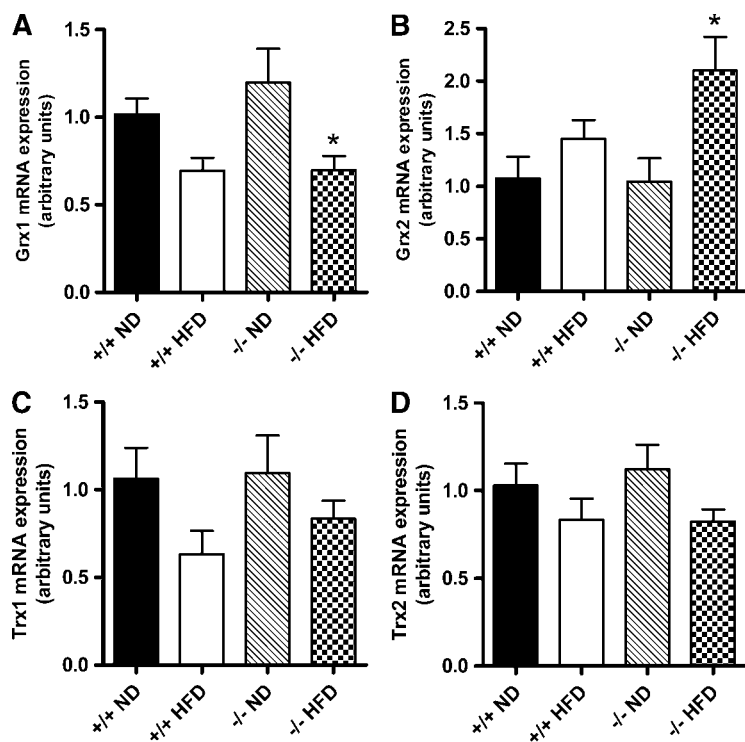
Fig. 5. Gene expression of four members of the thiol-disulfide oxidoreductase gene family, glutaredoxin-1 (Grx1; A), glutaredoxin-2 (Grx2; B), thioredoxin-1 (Trx1; C), and thioredoxin-2 (Trx2; D), in the aortic sinus region of control $(+/+)$ and Gpx1-deficient $(-/-)$ mice after 20 weeks of ND or HFD. For each gene investigated, gene expression is expressed relative to that in the control group fed ND, which is arbitrarily designated as 1. n = 5 for ND groups; n = 10 for HFD groups. Bars represent means ± SEM. * $P < 0.05$ versus $-/-$ ND group.

The second possibility is that other antioxidant enzymes compensate for the lack of Gpx1. Therefore, we investigated a selected range of antioxidants involved in the removal of H_2O_2 and lipid hydroperoxides that included all selenium-containing isoforms of Gpx as well as catalase. In particular, upregulation of phospholipid hydroperoxide Gpx4 may be important, as this isoform can act on lipoprotein-associated hydroperoxides of cholesteryl ester and phospholipids (27), in contrast to Gpx1. Our analyses, however, suggest that at the transcriptional level at least, Gpx4 was unaffected by the lack of Gpx1 in both the presence and absence of a HFD regime, implying that there is no increased contribution of this antioxidant enzyme to antiatherogenic mechanisms in the aortas of Gpx1-deficient mice. In addition, we were unable to detect significant changes in the expression of Gpx2 or Gpx3 after 20 weeks of HFD. The lack of compensatory response for Gpx2 and Gpx3 is less surprising, because in the mouse, the expression of Gpx2 is reported to be restricted to the gastrointestinal tract (42) and Gpx3 is present mainly in

secreted fluids of kidney (43). However, we noted that Gpx2 was the only isoform, at the transcriptional level at least, to increase in aortas of Gpx1-deficient mice fed ND. Therefore, it would be of interest to examine whether the observed increase in Gpx2 mRNA translates into increased Gpx2 protein, because this would suggest that Gpx2 is more widely distributed and its function is more diversified than originally described (42). Furthermore, one of us recently observed Gpx2 protein to be expressed in macrophage-derived foam cells of human carotid but not aortic root lesions in apolipoprotein E-deficient mice (R. Stocker, unpublished data). Similarly, the mRNA expression of catalase, the other major enzyme capable of removing H_2O_2, was unaltered by HFD in aortas in control and Gpx1-deficient mice independent of the diet used. These results suggest a limited role for this antioxidant in contributing to atherosclerosis and/or antiatherogenic mechanisms within Gpx1-deficient aortas. Our findings agree with previous data that catalase was unchanged in the aortas of Gpx1-deficient mice (33). Although catalase
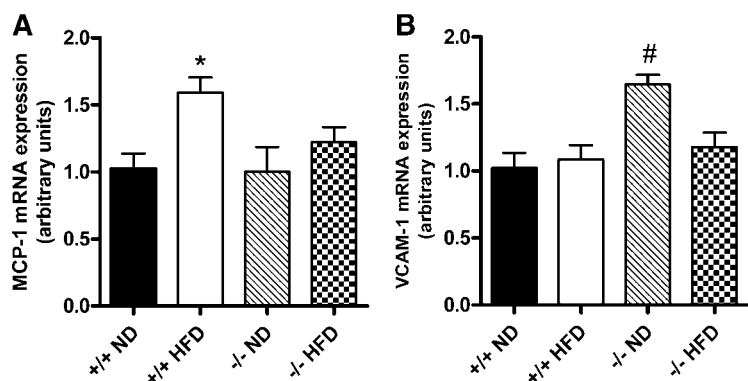
Fig. 6. Gene expression of known proinflammatory markers in the aortic sinus region of control $(+/+)$ and Gpx1-deficient $(-/-)$ mice after 20 weeks of ND or HFD. A: Monocyte chemoattractant protein-1 (MCP-1). B: Vascular cell adhesion molecule-1 (VCAM-1). For each gene investigated, gene expression is expressed relative to that in the control group fed ND, which is arbitrarily designated as 1. n = 5 for ND groups; n = 10 for HFD groups. Bars represent means ± SEM. * $P < 0.05$ versus $+/+$ ND group; # $P < 0.05$ versus $+/+$ ND group.

overexpression has been shown to limit lesion formation in apolipoprotein E-deficient mice (39), its role as a major player in the protection against atherosclerosis is questionable because catalase enzymatic activity is known to be particularly low in the vasculature (44).

The expression profiles of other enzymes implicated in cellular antioxidant defense were also investigated in this study. In particular, members of the thiol-disulfide oxidoreductase gene family, thioredoxin (45) and glutaredoxin (46), have been suggested to be particularly important in limiting cellular oxidative stress. One interesting observation of this study is that the expression of peroxiredoxin-6, glutaredoxin-1, and thioredoxin-1 and -2 remained unchanged after HFD in control mice, suggesting that these antioxidants do not respond to changes in the dietary intake of lipids, unlike Gpx1, which increased ~20-fold. In addition, the expression of these genes remained unchanged in Gpx1-deficient mice fed HFD, suggesting that these antioxidants may not compensate for the lack of Gpx1 in the presence of a lipid challenge. However, interestingly, we observed the expression of glutaredoxin-2 to be increased significantly in the sinus of Gpx1-deficient mice after 20 weeks of HFD. Glutaredoxin-2 is a recently discovered mitochondrial and nuclear glutaredoxin that differs from the more abundant cytosolic glutaredoxin-1 by its high affinity toward *S*-glutathionylated proteins and by being a substrate for thioredoxin reductase (47). It was shown recently to play an important role in the regulation of cell death at the mitochondrial checkpoint (48), and it attenuates apoptosis by preventing the release of cytochrome c (49). Also, glutaredoxin protein has been reported to increase in cultured smooth muscle cells after H_2O_2 treatment (46). Thus, upregulation of glutaredoxin-2 may limit atherogenesis in Gpx1-deficient mice by protecting against thiol-mediated protein damage, modulating apoptosis, and/or playing a role as an antiatherosclerotic signaling molecule, although these potential functions have yet to be determined (46). Finally, as our study of enzymatic antioxidants was not exhaustive, we cannot exclude the possibility that upregulation of other antioxidants limits oxidant stress induced by the lack of Gpx1 and that this may translate into differences in atherosclerosis. Furthermore, mRNA expression profiles may not accurately reflect protein levels or enzymatic activity; however, this approach was taken to maximize the amount of information attainable from the small amount of material available from the atherosclerosis-prone aortic sinus region of the mouse.

To gain additional mechanistic insight into the role of Gpx1 in HFD-induced atherogenesis, we investigated the gene expression of a number of proinflammatory markers. A HFD has been reported to induce an inflammatory-type response (50), and inflammation is considered to contribute to atherogenesis (29). Indeed, a characteristic feature of atherogenesis is enhanced monocyte adhesion to the endothelium, and the genes encoding MCP-1 and VCAM-1 are redox-sensitive and upregulated in response to proatherogenic stimuli (51, 52) and excess oxygen-derived free radicals (53, 54). Increased MCP-1 expression in con-

trol mice fed HFD for 20 weeks confirmed the involvement of this chemokine in our model. However, Gpx1 deficiency did not lead to a similar upregulation of MCP-1 expression, and this correlated with smaller lesions at this time point. Similarly, VCAM-1 was unaffected by the lack of Gpx1 after HFD feeding, although the involvement of this chemokine in this model of diet-induced atherosclerosis is less clear, because levels were unaffected in control mice fed HFD. These data suggest that known proinflammatory pathways are not activated in the absence of Gpx1, and this may have contributed to the lack of progression of atherosclerosis observed after 20 weeks of HFD in Gpx1-deficent mice.

An additional point of note is the heterogenicity in the response of various areas of the aorta to atherogenic stimuli. Indeed, Witting et al. (55) have shown that treatment with the antioxidant probucol increased lesions at the aortic root in apolipoprotein E-deficient mice, whereas it decreased lesion size in the descending aorta. Given that in the model used here, substantial atherosclerotic lesions are formed only in the aortic root region (23), our studies cannot rule out a role for Gpx1 in atherogenesis at other sites. It is also important to note that we did not characterize aortic cellularity or the extracellular matrix and thus cannot exclude the possibility of changes in these or other parameters in HFD-fed mice lacking Gpx1 that are not reflected by lipid staining. Furthermore, a potential role of Gpx1 in lesion formation may not be properly revealed unless the animals are challenged in a different, more severe, manner. In addition, extrapolation of data obtained with diet-induced mouse models of atherosclerosis to the human disease has clear limitations (56). Nevertheless, it is still valid to explore the impact of a deficiency of Gpx1 on atherosclerosis with the HFD model used here, as other models of atherosclerosis also have limitations. For example, the commonly used apolipoprotein E-deficient mouse is also known to develop diet-induced insulin resistance (57), which itself may complicate the interpretation of experimental data. Despite these limitations, genetic knockout of Gpx1 in more accelerated models of atherosclerosis, in which lesions are known to affect a greater region of the aorta, will shed light on the responses of other regions of the aorta to atherogenic stimuli in an environment of reduced antioxidant defense. We are currently investigating this paradigm in ongoing studies.

In conclusion, we have found that atherosclerosis is not accelerated in Gpx1-deficient mice after HFD at a defined region of the aorta, the aortic sinus. Therefore, our data challenge the notion that a specific deficiency in a key antioxidant enzyme increases susceptibility to atherosclerosis in mice fed HFDs.▪

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