Lack of the Antioxidant Enzyme Glutathione Peroxidase-1 Accelerates Atherosclerosis in Diabetic Apolipoprotein E–Deficient Mice

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Background—Recent clinical studies have suggested a major protective role for the antioxidant enzyme glutathione peroxidase-1 (GPx1) in diabetes-associated atherosclerosis. We induced diabetes in mice deficient for both GPx1 and apolipoprotein E (ApoE) to determine whether this is merely an association or whether GPx1 has a direct effect on diabetes-associated atherosclerosis.

Methods and Results—ApoE-deficient (ApoE−/−) and ApoE/GPx1 double-knockout (ApoE−/−/GPx1−/−) mice were made diabetic with streptozotocin and aortic lesion formation, and atherogenic pathways were assessed after 10 and 20 weeks of diabetes. Aortic proinflammatory and profibrotic markers were determined by both quantitative reverse-transcription polymerase chain reaction analysis after 10 weeks of diabetes and immunohistochemical analysis after 10 and 20 weeks of diabetes. Sham-injected nondiabetic counterparts served as controls. Atherosclerotic lesions within the aortic sinuses, as well as arch, thoracic, and abdominal lesions, were significantly increased in diabetic ApoE−/−/GPx1−/− aortas compared with diabetic ApoE−/− aortas. This increase was accompanied by increased macrophages, α-smooth muscle actin, receptors for advanced glycation end products, and various proinflammatory (vascular cell adhesion molecule-1) and profibrotic (vascular endothelial growth factor and connective tissue growth factor) markers. Quantitative reverse-transcription polymerase chain reaction analysis showed increased expression of receptors for advanced glycation end products (RAGE), vascular cell adhesion molecule-1, vascular endothelial growth factor, and connective tissue growth factor. Nitrotyrosine levels were significantly increased in diabetic ApoE−/−/GPx1−/− mouse aortas. These findings were observed despite upregulation of other antioxidants.

Conclusions—Lack of functional GPx1 accelerates diabetes-associated atherosclerosis via upregulation of proinflammatory and profibrotic pathways in ApoE−/− mice. Our study provides evidence of a protective role for GPx1 and establishes GPx1 as an important antiatherogenic therapeutic target in patients with or at risk of diabetic macrovascular disease. (Circulation. 2007;115:2178-2187.)

Key Words: cardiovascular diseases ■ aorta ■ atherosclerosis ■ diabetes mellitus ■ antioxidants ■ free radicals

Diabetes mellitus (DM) is a major risk factor for the development of atherosclerosis, with a 2- to 4-fold higher incidence of cardiovascular disease in diabetic patients than in the general population.1 Other known risk factors, such as dyslipidemia, hyperglycemia, hypertension, and obesity, only partly explain the more advanced lesions2 and increased incidence observed in these patients. Although the underlying mechanisms that accelerate diabetic atherosclerosis remain unknown, various pathways have been implicated, including the biochemical process of advanced glycation3 and, in particular, the receptor for advanced glycation end products (AGEs). RAGE.4 Furthermore, various proteins implicated in the atherosclerotic process are upregulated in the diabetic condition, including the adhesion molecule vasa...
cular cell adhesion molecule-1 (VCAM-1), the chemokine monocyte chemotactic protein-1, and the profibrotic growth factor connective tissue growth factor (CTGF). In addition, strong evidence suggests the involvement of reactive oxygen species (ROS) derived from the hyperglycemia-driven increase in mitochondrial electron transport chain activity, glucose autoxidation, and enzymes such as NAD(P)H oxidase. ROS, in turn, are known to upregulate a number of proatherogenic processes, such as monocyte infiltration, platelet activation, smooth muscle cell (SMC) migration, cell adhesion, release of CTGF, and increased production of AGEs. Indeed, many of these processes are enhanced in a murine model of DM-associated atherosclerosis, Importantly, a heightened state of oxidative stress has been observed in diabetic patients and postulated to play a role in a range of diabetic complications. Eukaryotic cells have evolved an extensive array of antioxidant defenses to regulate the flux of ROS and to limit oxidative damage. Blood vessels are known to express antioxidant enzymes to counteract oxidant stress. However, reductions in antioxidant defense, which lead to increased ROS accumulation, can elicit pathophysiological consequences. Recent evidence from apolipoprotein (Apo) E–deficient mice suggest that the levels of several antioxidant enzymes decline during atherogenesis, which implies a link between reduced antioxidant capacity and increased lesion formation. Conversely, overexpression of the antioxidant catalase, known to remove hydrogen peroxide, reduced the severity of lesions in ApoE-deficient mice, which implies a role for hydrogen peroxide–scavenging enzymes in atherosclerotic processes. However, limited information is available on the involvement of individual antioxidant enzymes in DM-associated atherosclerosis.

Glutathione peroxidase-1 (GPx1) is a major and ubiquitously expressed antioxidant enzyme present in the cytosol and mitochondria. It is involved in the detoxification of hydrogen and lipid peroxides and acts as a peroxynitrite reductase. In the absence of this antioxidant enzyme, a buildup of ROS ensues that is known to damage DNA, proteins, and lipids. Clinical evidence now suggests a potential role for GPx1 in DM-associated atherogenesis. Polymorphisms identified within the GPx1 gene that result in reduced GPx1 activity have been linked with increased intima-media thickness of carotid arteries and an increased risk of cardiovascular and peripheral vascular disease in type 2 DM patients. Furthermore, additional studies suggest a protective role for GPx1 in the atherogenic process per se. For example, reductions in red blood cell GPx1 activity were associated with an increased risk of cardiovascular events in a prospective cohort study, and atherosclerotic plaques of patients with carotid artery disease have reduced GPx1 activity. In animal studies, reduced GPx1 expression increased cell-mediated oxidation of LDL and decreased the bioavailability of nitric oxide, leading to endothelial dysfunction. These findings imply that GPx1 is a key enzyme for the protection of vessels against oxidative stress and atherogenesis and that GPx1 may be of particular importance in the highly prooxidant diabetic environment. However, to date, no study has directly tested the impact of reduced GPx1 function on the development of atherosclerosis in a diabetic context. Accordingly, the aims of the present study were (1) to evaluate the effects of a lack of GPx1 on the development of DM-associated atherosclerosis and (2) to determine whether a lack of GPx1 impacts on known pathways implicated in DM-associated atherosclerosis.

**Methods**

**Animal Groups and Experimental Design**

GPx1-deficient mice on a C57Bl/J6 genetic background and described by us previously were mated with C57Bl/J6 ApoE-deficient (ApoE–/–) mice to establish a line of ApoE–/–GPx1–/– double-knockout mice. ApoE–/– mice were generated through the breeding program and were maintained as a separate line. All studies were performed in male age-matched ApoE–/– or ApoE–/–GPx1–/– mice.

Eight-week-old ApoE–/– and ApoE–/–GPx1–/– mice were rendered diabetic by 2 intraperitoneal injections of streptozotocin (Sigma-Aldrich, St. Louis, Mo) on consecutive days, at a dose of 100 mg · kg–1 · d–1. Sham-injected ApoE–/– and ApoE–/–GPx1–/– mice served as nondiabetic controls. Nonfasted blood glucose readings were measured every 3 to 4 weeks from the tail vein. Animals had unrestricted access to water and standard mouse chow. All studies were performed with approval of the Institutional Ethics Committee.

Animals were maintained for 20 weeks (20-week study) for enface aortic lesion analysis. In addition, to assess the status of other antioxidants and various pathways implicated in the progression of atherosclerosis, separate groups were assessed after 2 or 10 weeks of DM. Lesions were quantitated within the aortic sinus at both the 10- and 20-week time points. The study specifically involved enface assessment after 20 weeks of DM on the basis of previous studies in our laboratory in which total plaque area was shown to be significantly increased in diabetic ApoE–/– mice compared with nondiabetic controls. We specifically chose to investigate the expression of known proatherogenic markers after 10 weeks of DM to establish whether lack of GPx1 affected proatherogenic pathways at an earlier stage, thereby gaining valuable information about potential molecular pathways involved in the progression toward highly developed lesions. Because oxidative stress and activation of redox-sensitive transcription factors have been shown to occur within hours after glucose treatment, and therefore as an early event of the diabetic milieu, we also believed that analysis at the earlier 2-week time point would give additional insight into the effects of a lack of GPx1.

**Blood Sampling, Plasma Biochemistry, and Tissue Collection**

Mice were anesthetized by an intraperitoneal injection of 2,2,2-tribromoethanol (Sigma Chemical Co., St. Louis, Mo) after food was withheld for 4 hours. The thoracic cavity was opened rapidly and blood drawn by direct puncture of the right ventricle. Blood samples were placed into heparinized tubes and centrifuged at 2500 rpm, and plasma was frozen at −20°C until analyzed.

Plasma glucose, cholesterol, HDL, and triacylglycerols were measured with commercial enzymatic kits. The Friedewald calculation was used to determine levels of LDL.

Hearts with aortas attached were rapidly removed and placed in cold saline. The aorta was severed close to the heart and cleaned of extraneous fat under a dissecting microscope. Cleaned aortas from the 2- and 10-week study were snap-frozen in liquid nitrogen and stored at −80°C until analyzed.

**Evaluation of Atherosclerotic Lesions**

Atherosclerosis was assessed by 2 approaches, first by the enface technique described by us previously to determine total and regional aortic plaque distribution, and second, by cross-sectional assessment through the aortic sinus. After enface analysis, aortic tissue was embedded in paraffin, and cross-sectional serial sections 3-μm thick were prepared for immunohistochemical analysis. Lesions within the
aortic sinus region were assessed on the basis of the method of Paigen et al, and described by us previously. Briefly, 10-μm-thick cryostat sections, selected 80 μm apart and covering 320 μm of the sinus, were stained with oil red O to detect lesions. Images were quantitated with Optimas software (Media Cybernetics, version 6.2; Bethesda, Md). For each mouse, lesion size (μm²) was determined from the average of 5 cross sections, and the results of each group are expressed as lesion size (μm²) ± SEM. (See also the expanded Methods section in the online Data Supplement).

**Immunohistochemistry**

Serial aortic paraffin sections were stained for α-smooth muscle actin (α-SMA), RAGE, the macrophage marker F4/80, nitrotyrosine, gp91phox, CTGF, and vascular endothelial growth factor (VEGF). Frozen aortic sinus sections were stained for VCAM-1. Primary and secondary antibodies and dilutions are described in Data Supplement Table I. Immunohistochemical methods were described by us previously (see also the expanded Methods section in the Data Supplement). Images were visualized under light microscopy and quantitated with Optimas (Media Cybernetics, version 6.2). Analysis was performed in a blinded fashion. On average, 3 sections were assessed per mouse, and 7 to 9 mice were analyzed per group. Results were calculated as percentage of positively stained tissue in the aortic media (for all except VCAM-1, for which the percentage of positive staining was determined within the sinus region) and expressed relative to nondiabetic ApoE−/− mice that were arbitrarily assigned a value of 1.

**Quantitative Reverse-Transcription Polymerase Chain Reaction**

Total RNA extraction, preparation of DNA-free RNA, and RNA reverse transcription have been described by us previously. Gene expression of VCAM-1; CTGF; VEGF; RAGE; α-SMA; nuclear factor (NF)-κB subunit p65; the antioxidant enzymes GPx1, GPx3, GPx4, catalase, and superoxide dismutase-1 (Sod1) and -2 (Sod2); and the NAD(P)H oxidase subunit gp91phox were analyzed by quantitative reverse-transcription polymerase chain reaction as described by us previously. (See also online Data Supplement Methods and Table II, which describe probes and primers for VEGF, Sod1, Sod2, gp91phox, NF-κB, and RAGE).

**Immunoblot Analysis of Individual Aortas**

Individual aortas were homogenized in 20 mmol/L cold HEPES buffer containing 1 mmol/L EGTA, 210 mmol/L mannitol, and 70 mmol/L sucrose. Cytosolic preparations were prepared by 2-step centrifugation; the first at 150,000g for 5 minutes followed by a 10,000g spin for 15 minutes. Protein concentration was determined, and equivalent amounts of protein were loaded per lane. We used rabbit polyclonal anti-GPx1 antibody, anti-Cu/Zn superoxide dismutase (SOD) antibody, and anti-catalase antibody (all from Abcam Ltd, Cambridge, United Kingdom) as the primary antibodies. Pierce (Rockford, Ill) ImmunoPure goat anti-rabbit IgG, peroxidase conjugated, was used as secondary antibody. Mouse monoclonal anti-β-actin antibody (Abcam Ltd; ab6276) and goat anti-mouse IgG, peroxidase conjugated (Dako, Glostrup, Denmark), were used as primary and secondary antibodies to detect β-actin. Proteins were detected by ECL (Pierce SuperSignal West Pico Chemiluminescence Substrate) and visualized on a ChemiDoc documentation system (Bio-Rad Laboratory, Milan, Italy). Band intensities were determined by densitometry. Four aortas were analyzed per group, and the results are expressed as mean±SEM (see also Data Supplement Methods section).

**Statistical Analysis**

Data were analyzed by 2-way ANOVA with diabetic status and GPx genotype as the 2 variables. Pairwise multiple comparisons were made with the Student-Newman-Keuls method to detect significant differences between groups (SigmaStat version 2.03, Systat Software, San Jose, Calif). Results are expressed as mean±SEM. P<0.05 was considered statistically significant.

The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.

**Results**

**Phenotypic Assessment of ApoE−/−GPx1−/− Mice and Metabolic Parameters**

We generated mice lacking both GPx1 (GPx1−/−) and ApoE (ApoE−/−). Double-knockout mice (ApoE−/−GPx1−/−) appeared healthy, bred normally, and exhibited standard mendelian transmission of both mutant alleles. No obvious pathological changes were observed in any organ investigated.

In the absence of DM, lack of GPx1 did not affect the lipid profile of any of the major lipoprotein groups investigated in sham-injected ApoE−/− mice analyzed after 20 weeks, as is shown in Data Supplement Table III. Similarly, body weight and...
plasma glucose were unaffected by the lack of GPx1 in sham-injected ApoE+/−GPx1+/− mice (Data Supplement Table III).

In mice administered streptozotocin, nonfasted tail-vein blood glucose levels were significantly increased for the duration of the study compared with sham-injected animals (data not shown). Importantly, similar blood glucose levels were achieved for diabetic ApoE−/− and ApoE−/−GPx1−/− mice, thereby eliminating differences in hyperglycemia as potentially contributing to experimental outcomes. Plasma glucose levels from fasted animals were also significantly and similarly increased in diabetic ApoE−/− and ApoE−/−GPx1−/− mice compared with nondiabetic counterparts at the conclusion of the 10-week (P<0.01; data not shown) and 20-week (P<0.01; Data Supplement Table III) studies. Body weights were not significantly affected by DM in either group, as shown in Data Supplement Table III. Twenty weeks of DM did not affect triglycerides, HDL, LDL, or the LDL/HDL ratio of ApoE−/− mice in the present study. A trend toward elevated total cholesterol levels occurred in diabetic ApoE−/− mice, although this did not reach significance (P=0.06). Similarly, lack of GPx1 did not affect lipid profiles of diabetic ApoE−/−GPx1−/− mice after either 10 (data not shown) or 20 (Data Supplement Table III) weeks of DM.

**Assessment of Aortic Atherosclerotic Lesions**

**En Face Analysis of Aortic Tree**

In the absence of DM, lack of GPx1 did not affect the regional or total plaque deposition in aortas of sham-injected ApoE−/− mice (Figure 1A, 1B, 1E, and 1F). Diabetes was associated with a 2.5-fold increase in total plaque in ApoE−/− mice (P<0.01; Figure 1C and 1E). Regional evaluation revealed a 3.0-fold increase in the highly susceptible arch region (P<0.001) together with a significant increase in thoracic plaque (P<0.05) in diabetic ApoE−/− aortas compared with nondiabetic ApoE−/− aortas (Figure 1C and 1F). A similar trend, albeit not significant, was observed in the abdominal region of these aortas. Diabetic mice lacking GPx1 induced DM. Importantly, lack of GPx1 augmented this further 2.0-fold increase in total plaque compared with diabetic ApoE−/− mice (P<0.001; Figure 1D and 1E). Similarly, the aortic arch (P<0.001), thoracic (P<0.01), and abdominal regions (P<0.01) showed significant increases in plaque deposition in diabetic ApoE−/−GPx1−/− compared with diabetic ApoE−/− aortas (Figure 1D and 1F).

**Aortic Sinus Region**

In the absence of DM, lack of GPx1 did not affect lesion size or composition within the sinus region of mice, 10 or 20 weeks after sham injection (P>0.05; Figure 2A, 2B, and 2E and Figure 2F, 2G, and 2I, respectively). An approximately 2.5- to 3-fold increase in lesion size was observed within the aortic sinus of ApoE−/− mice after 10 (P<0.01; Figure 2A, 2C, and 2E) and 20 (P<0.001; Figure 2F, 2H, and 2J) weeks of streptozotocin-induced DM. Importantly, lack of GPx1 augmented this further 1.5- to 2.5-fold after 10 (P<0.001; Figure 2C, 2D, and 2E) and 20 (P<0.001; Figure 2H, 2I, and 2J) weeks of DM.

**Proatherogenic Markers in the Absence of DM**

In the absence of DM, lack of GPx1 did not significantly affect any of the proatherogenic markers investigated in the present study, either at the transcriptional or protein level (P>0.05 for all; VCAM-1: Figure 3E and 3F; CTGF: Figure 4E and 4F; VEGF: Figure 4K and 4L; α-SMA: Figure 5A and 5B; RAGE: Figure 5C and 5D; and F4/80: Figure 5E).

**Proatherogenic Markers in the Presence of DM**

VCAM-1 gene expression (Figure 3E) and protein levels (Figure 3A through 3D and 3F) were significantly increased (P<0.01) within the aortic sinus of diabetic ApoE−/−GPx1−/− mice compared with diabetic ApoE−/− controls after 10 weeks of DM. Interestingly, VCAM-1 levels were mainly increased within the
vascular tissue. Consequently, we investigated the expression of (1) GPx1; (2) the extracellular lipid and hydrogen peroxide–reducing isomerase, GPx3; (3) the phospholipid hydroperoxide-reducing isomerase, GPx4; (4) the peroxisomal hydrogen peroxide–specific reducing enzyme, catalase; and (5) the cytosolic and mitochondrial isomorphs (Sod1 and Sod2, respectively) of the SOD enzyme family.

DM was associated with a significant 2-fold increase in GPx1 mRNA levels in ApoE^{-/-} aortas (Figure 6A; \( P<0.001 \)), most likely as a response to DM-induced increases in oxidative stress. Homozygous disruption of the GPx1 gene resulted in significant reductions in GPx1 mRNA expression in both diabetic and nondiabetic ApoE^{-/-}/GPx1^{-/-} aortas, in agreement with previous data. The approximately 10% to 20% residual GPx1 expression in GPx1-deficient aortas most likely reflects background or nonspecific primer amplification, because we were unable to detect any GPx1 protein after immunoblot analysis of individual ApoE^{-/-}/GPx1^{-/-} aortas (Figure 6A), consistent with homozygous knockout of the GPx1 gene. Diabetic ApoE^{-/-}/GPx1^{-/-} aortas also showed significant increases in GPx3 expression, with similar trends, albeit nonsignificant, for GPx4 (Figure 6A), catalase, and Sod2 (Figure 6B) compared with nondiabetic ApoE^{-/-} controls, most likely a consequence of enhanced oxidative stress known to accompany DM. Similarly, GPx3, GPx4, catalase, and Sod1 and Sod2 expression was significantly increased in diabetic ApoE^{-/-}/GPx1^{-/-} aortas compared with nondiabetic counterparts (Figure 8F; \( P<0.05 \) and \( P<0.01 \), respectively) and that these changes were already apparent after 2 weeks of DM.

**Prooxidant NAD(P)H Oxidase and NF-κB Gene Expression**

We were interested in the potential impact that lack of GPx1 might have on the expression of the superoxide-generating NADPH oxidase within a diabetic milieu, because lipid peroxides are implicated in the upregulation of NADPH oxidase. Lack of GPx1 did not significantly affect gene expression or protein levels of gp91phox (the subunit associated with phagocytic NADPH oxidase) in the absence of DM (Figure 7E and 7F). Gene expression and protein levels were significantly increased in diabetic ApoE^{-/-}/Gpx1^{-/-} compared with diabetic ApoE^{-/-} aortas (Figure 7E and 7F; \( P<0.01 \) and \( P<0.05 \), respectively). In light of recent data that NF-κB mediates oxidant-induced upregulation of NADPH oxidase, we also investigated the expression of the p65 subunit of NF-κB. We found p65 mRNA expression to be significantly increased in diabetic ApoE^{-/-}/Gpx1^{-/-} compared with nondiabetic ApoE^{-/-} aortas (Figure 8F; \( P<0.05 \)) and importantly, significantly increased a further 2.5-fold in diabetic ApoE^{-/-}/Gpx1^{-/-} aortas (\( P<0.001 \)) compared with diabetic ApoE^{-/-} aortas.

**Antioxidant Gene Expression and Immunoblot Analysis**

Because DM and atherosclerosis can alter the expression of various antioxidant enzymes, and deletion of 1 enzyme such as GPx1 may result in compensatory changes in other antioxidant enzymes, we investigated the expression of a range of antioxidants with the potential to remove ROS in vessel wall (Figure 3D). Similarly, the macrophage marker F4/80 was significantly increased in diabetic ApoE^{-/-}/Gpx1^{-/-} aortas compared with diabetic ApoE^{-/-} controls (\( P<0.01 \); Figure 5E). Gene expression and protein levels were significantly increased for CTGF (Figure 4E and 4F; \( P<0.001 \) and \( P<0.01 \), respectively), VEGF (Figure 4K and 4L; \( P<0.01 \) and \( P<0.05 \), respectively), α-SMA (Figure 5A and 5B; \( P<0.001 \) and \( P<0.05 \), respectively), and RAGE (Figure 5C and 5D; \( P<0.01 \)) in diabetic ApoE^{-/-}/Gpx1^{-/-} compared with diabetic ApoE^{-/-} aortas. Photomicrographs for α-SMA, RAGE and F4/80 immunostaining are provided in Data Supplement Figure I.
Detection of Nitrotyrosine in Aortas

Lack of GPx1 caused a significant increase in nitrotyrosine levels in diabetic ApoE$^{-/-}$ Gpx1$^{-/-}$ aortas (Figure 8A through 8E; $P<0.01$ versus nondiabetic counterparts and diabetic ApoE$^{-/-}$), suggestive of increased peroxynitrite-mediated damage to proteins in these aortas. No change was observed in nondiabetic ApoE$^{-/-}$ Gpx1$^{-/-}$ or diabetic ApoE$^{-/-}$ aortas.

**Discussion**

In the present study, we demonstrate that a deficiency in GPx1 accelerates DM-associated atherosclerosis in the ApoE-knockout mouse. The present data are highly supportive of a recent clinical study in which diabetic patients with reduced GPx1 function, as a consequence of polymorphisms within the GPx1 gene, were shown to have an increased risk for cardiovascular disease. The present study has also shown that accelerated DM-driven atherosclerosis is accompanied by the presence of increased nitrotyrosine, a marker of free radical damage, and by transcriptional changes of important inflammatory and profibrotic pathways. These molecular changes translated into increased levels of α-SMA, profibrotic CTGF and VEGF, proinflammatory VCAM-1, and RAGE and the increased presence of proinflammatory macrophages. The present study therefore unequivocally links a lack of the important intracellular ROS-removing antioxidant enzyme GPx1 with increased DM-associated atherosclerosis and identifies areas in which antioxidant defense, and GPx1 in particular, plays an important role in regulating atherogenic processes within a diabetic milieu.

The present study also shows that GPx1 does not appear as important in preventing atherosclerosis in ApoE$^{-/-}$ mice in the absence of DM. This difference between diabetic and nondiabetic mice may arise as a consequence of the higher prooxidant environment of DM. Because hyperglycemia is known to greatly increase oxidative stress through various mechanisms, the beneficial antioxidant function of GPx1 is likely to be of particular importance within a diabetic context. However, the impact of a lack of GPx1 on atherosclerosis in a nondiabetic context needs to be more fully explored in models that more directly address atherosclerosis than that used in the present study. We have recently shown that high-fat feeding of mice results in greatly increased GPx1 expression, which most likely is a consequence of the enhanced oxidative stress known to accompany such diets. Therefore, feeding prooxidant, proatherogenic diets should give a clearer indication of GPx1 function in the protection against atherosclerosis in ApoE$^{-/-}$ mice in the absence of DM.

To expand on the mechanisms whereby a lack of GPx1 enhances atherosclerosis in a diabetic milieu, a number of cellular and molecular processes linked to DM-associated atherosclerosis were examined. First, we investigated the impact of reduced GPx1 function on the presence of macrophages and SMCs, because these cell types are integral components of plaque and are known to be increased in DM-associated athero-
staining for nitrotyrosine is suggestive of increased ROS in diabetic ApoE−/−GPx1−/− aortas and is supportive of this notion. In agreement with the findings of Wang et al., vascular SMC migration may involve the growth factor VEGF, because we show significantly increased VEGF in ApoE−/−GPx1−/− aortas.

To further explore the underlying molecular mechanisms that may accelerate proinflammatory cell migration into the vascular wall of diabetic ApoE−/−GPx1−/− mice, we investigated the expression of an important mediator of the inflammatory response, namely, the cell adhesion molecule VCAM-1. Furthermore, we chose to focus on VCAM-1 based on strong supportive data that VCAM-1 is a redox sensitive and upregulated by ROS. Indeed, in the present study, lack of GPx1 led to increased VCAM-1 mRNA and protein levels after 10 weeks of DM, which suggests that lack of GPx1 directly affected transcription and translation of this proinflammatory molecule in the progression toward highly developed lesions. Given the known antioxidant functions of GPx1, this most likely occurred via ROS-related mechanisms. Indeed, a number of pathways linked to the upregulation of VCAM-1 involve ROS. First, angiotensin II, the biologically active peptide generated by the renin-angiotensin system, upregulates VCAM-1 via ROS-mediated mechanisms. This effect was inhibited by a glutathione peroxidase mimic and enhanced by the catalase inhibitor aminotriazole. Similarly, RAGE upregulates VCAM-1 via ROS-mediated mechanisms. Therefore, a number of pathways may be activated by the lack of GPx1 that in turn increase VCAM-1 and contribute to proinflammatory cell migration. It is therefore also of particular interest that we found RAGE expression, both at the transcriptional and protein level, to be increased by a lack of GPx1 in diabetic ApoE−/− aortas. The present data are consistent with and add to previous studies that demonstrate ROS-mediated upregulation of RAGE within the vasculature. In light of recent data that lipid peroxides upregulate the proinflammatory molecule VCAM-1, we propose that increased RAGE, as a consequence of reduced GPx1 function, may contribute to the accelerated atherosclerosis detected in these animals, possibly via upregulation of VCAM-1.

In agreement with a previous study, the present study has once again shown the involvement of the profibrotic growth factor CTGF in DM-associated atherosclerosis. In addition, the present study now suggests that upregulation of CTGF is mediated in part via ROS, because lack of GPx1 further upregulated this cytokine in diabetic ApoE−/− mice, and the present study is supportive of Ruperez et al., who observed a significant ROS-mediated increase in CTGF expression within rat SMCs.

In light of recent data that lipid peroxides upregulate the superoxide-producing enzyme NADPH oxidase via the transcription factor NF-κB, we investigated whether lack of GPx1 affects NADPH oxidase. Indeed, we demonstrate for the first time within the vasculature of diabetic mice that reduced GPx1 leads to the increased expression of gp91phox, one of the major

The present study showed augmented staining for both cell types in the aortas of diabetic ApoE−/−GPx1−/− mice, which suggests that GPx1 plays an important role in limiting macrophage infiltration and SMC migration during DM-associated atherogenic processes. It is most likely that GPx1 limits such cellular invasion via its antioxidant function in the removal of DM-induced ROS, especially because ROS have been implicated in enhancing monocyte chemotaxis and SMC migration. In particular, Wang et al. demonstrated evidence for VEGF-mediated vascular SMC migration via ROS-mediated activation of NF-κB, whereas pretreatment of these cells with the antioxidant N-acetylcysteine reduced ROS and cellular migration. Indeed, the present data, which show increased

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subunits of NADPH oxidase, at both the mRNA and protein level. The present data therefore support the notion that NADPH oxidase is regulated by ROS.\textsuperscript{34} We also show increased expression of the p65 subunit of NF-\kappaB. We speculate that lack of GPx1 within a diabetic context sets into motion a cyclical event that initially includes the formation of ROS, such as lipid peroxides. These ROS then upregulate NADPH oxidase, possibly via the ROS-sensitive transcription factor NF-\kappaB, and in so doing increase superoxide formation. In turn, superoxide, the substrate of the SOD enzymes, is converted into hydrogen and lipid peroxide, thereby completing and enhancing the cycle. Indeed, the present data support this concept, because prooxidants (NADPH oxidase) and antioxidants (Sod1 and Sod2) upstream of GPx1 are affected by the lack of GPx1 in a diabetic milieu. The present study has shown that these antioxidant changes occur early after the initiation of DM and are still evident after prolonged exposure to the diabetic stimulus, as assessed by measurement of these enzymes at both the gene and protein level. In addition, superoxide can interact with nitric oxide, forming peroxynitrite, the potent cytotoxin known to attack various biomolecules in the vascular endothelium, vascular smooth muscle, and myocardium.\textsuperscript{38} Indeed, the present data showing enhanced staining of nitrotyrosine in GPx1-deficient diabetic aortas suggest that peroxynitrite may contribute to atherogenic mechanisms in this model, which is consistent with the notion that GPx1 functions as a peroxynitrite reductase.\textsuperscript{20}

In summary, the present study has demonstrated that lack of GPx1 enhances atherosclerosis in a diabetic setting. This effect was associated with increased staining for SMCs and macrophages, consistent with increased SMC migration and macrophage infiltration. Furthermore, a range of molecules implicated in the progression and development of atherosclerosis, including adhesion mole-

![Figure 6](http://circ.ahajournals.org/)

**Figure 6.** A, Aortic GPx1 expression is shown in the left panel (top, after 2 weeks of DM by immunoblot analysis; bottom, after 10 weeks of DM, by quantitative reverse-transcription polymerase chain reaction analysis). Quantitative reverse-transcription polymerase chain reaction analysis for GPx3 and GPx4 is shown on the right. GPx1 is significantly increased in diabetic ApoE\textsuperscript{−/−} aortas; immunoblotting fails to detect GPx1 protein in ApoE\textsuperscript{−/−} GPx1\textsuperscript{−/−} aortas. GPx3 and GPx4 expression is significantly increased in diabetic double knockout (dKO) compared with diabetic ApoE\textsuperscript{−/−} aortas. For each gene investigated, gene expression is shown relative to the nondiabetic ApoE\textsuperscript{−/−} group, which is arbitrarily designated as 1. GPx1: ***P<0.001 vs nondiabetic ApoE\textsuperscript{−/−}; ***P<0.001 vs diabetic ApoE\textsuperscript{−/−}. GPx3 and 4: **P<0.01 and ***P<0.001 vs both nondiabetic groups; #P<0.05 vs diabetic ApoE\textsuperscript{−/−}. Bars represent mean±SEM (n=4 to 10 aortas/group). 1=nondiabetic ApoE\textsuperscript{−/−} aorta; 2=nondiabetic dKO aorta; 3=diabetic ApoE\textsuperscript{−/−} aorta; 4=diabetic dKO aorta. B, Quantitative reverse-transcription polymerase chain reaction analysis of Sod1, Sod2, and catalase 10 weeks after sham injection (nondiabetic; ND) or streptozotocin-induced DM (D). For each gene investigated, gene expression is shown relative to the nondiabetic ApoE\textsuperscript{−/−} group, which is arbitrarily designated as 1. Bars represent mean±SEM (n=4 to 10 aortas/group). ***P<0.001, *P<0.05 vs both nondiabetic groups; #P<0.01 vs diabetic ApoE\textsuperscript{−/−}. C, Immunoblot analysis of Sod1 and catalase 10 weeks after sham injection or streptozotocin-induced DM. Top panel of each shows a representative immunoblot. 1=nondiabetic ApoE\textsuperscript{−/−} aorta; 2=nondiabetic dKO aorta; 3=diabetic ApoE\textsuperscript{−/−} aorta; 4=diabetic dKO aorta. Below each are the results after densitometric analysis (n=4 aortas/group). Results are normalized against β-actin and expressed relative to the nondiabetic ApoE\textsuperscript{−/−} group, which is arbitrarily designated as 1. Bars represent mean±SEM. *P<0.05 vs nondiabetic ApoE\textsuperscript{−/−} group. a.u. indicates arbitrary units.
molecules, growth factors, and RAGE, were increased by the absence of GPx1. These findings are consistent with recent clinical data implicating antioxidant defense, and GPx1 in particular, as playing a pivotal role in the progression of DM-associated atherosclerosis.

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Dr de Haan is a recipient of a research grant from Merck Research Laboratories; Dr Allen is a holder of a joint Australian National Health and Medical Research Council (NHMRC)/Diabetes Australia Career Development Grant; and Dr Calkin is a holder of an Australian National Health and Medical Research Council postdoctoral fellowship. This work is supported by a Juvenile Diabetes Research Foundation grant, awarded to Dr Cooper.

Disclosures
At the time of the present study, Dr Kola was a Senior Vice President for Basic Research at Merck & Co, Inc. Merck & Co has substantial revenues from drugs that treat dyslipidemias; however, no activities fall within the specific area covered by this article. The remaining authors report no conflicts.

References


**CLINICAL PERSPECTIVE**

In the present study, we show the importance of the abundant cytosolic and mitochondrial antioxidant enzyme glutathione peroxidase-1 (GPx1) in limiting diabetes-associated atherosclerosis. Lack of GPx1 is shown to accelerate lesion formation within the entire aortic tree and the susceptible aortic sinus, as well as to affect known proatherosclerotic and proinflammatory pathways. In particular, we demonstrate increased cellular involvement (macrophages and smooth muscle cells), increased expression of inflammatory (vascular cell adhesion molecule-1) and prosclerotic (vascular endothelial growth factor and connective tissue growth factor) cytokines, and upregulation of receptors for advanced glycation end products in diabetic apolipoprotein E−/−GPx1−/− double-knockout mice. This occurred despite upregulation of other antioxidants (GPx3, GPx4, Sod1, Sod2, and catalase), which highlights the importance of GPx1 in limiting oxidative events in the diabetic milieu. In addition, we show increased staining for a marker of free radical damage, nitrotyrosine, which emphasizes the importance of GPx1 as a peroxynitrite reductase. These findings are particularly important in light of recent clinical studies that have focused attention on GPx1 in atherosclerosis. For example, a patient cohort showed blood GPx1 activity to be the strongest predictor of cardiovascular disease, whereas reduced GPx1 activity was associated with increased cardiovascular disease in a diabetic population. These clinical studies, although clearly of significant diagnostic importance, offered no functional role for GPx1 in atherogenesis. The present study has clearly shown that lack of GPx1 affects important proatherogenic pathways in a diabetic setting, which suggests a significant antiatherogenic role for GPx1 and makes this antioxidant an important therapeutic target worthy of further study.
Lack of the Antioxidant Enzyme Glutathione Peroxidase-1 Accelerates Atherosclerosis in Diabetic Apolipoprotein E–Deficient Mice
Paul Lewis, Nada Stefanovic, Josefa Pete, Anna C. Calkin, Sara Giunti, Vicki Thallas-Bonke, Karin A. Jandeleit-Dahm, Terri J. Allen, Ismail Kola, Mark E. Cooper and Judy B. de Haan

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Supplemental Figure and Material and Methods for Lewis et al., AHA/2006/664250/R3

Supplemental Figure 1
FIGURE LEGEND FOR SUPPLEMENTAL Figure 1:
Representative photomicrographs show staining of α-smooth muscle actin (α-SMA) (A-D); receptors for advanced glycation end products, RAGE (E-H); and the macrophage marker F4/80 (I-L) within the aortic medial layer of non-diabetic ApoE\(^{-/-}\) (A,E,I); non-diabetic ApoE\(^{-/-}\)GPx1\(^{-/-}\) (B,F,J); diabetic ApoE\(^{-/-}\) (C,G,K); and diabetic ApoE\(^{-/-}\)GPx1\(^{-/-}\) (D,H,L) aortas, 20 weeks after sham-injection or streptozotocin-induced diabetes. α-SMA, RAGE and F4/80 are detected as brown staining within the aortic medial layer. Sections are counterstained with hematoxylin. Magnification X 100.

SUPPLEMENTAL Table 1: Primary and secondary antibodies used in immunohistochemical analyses.

<table>
<thead>
<tr>
<th>Primary antibody (Immunohistochemistry)</th>
<th>Manufacturer and Dilution</th>
<th>Secondary Antibody</th>
<th>Manufacturer and Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monoclonal mouse anti-human α-smooth muscle actin (α-SMA)</td>
<td>Dako; 1:4000</td>
<td>Biotinylated anti-mouse immunoglobulin</td>
<td>Vector Lab, Burlingame, CA, USA; 1:250</td>
</tr>
<tr>
<td>Polyclonal goat anti-human receptor for advanced glycated endproducts (RAGE)</td>
<td>Chemicon; 1:4000</td>
<td>Biotinylated anti-goat immunoglobulin</td>
<td>Vector Lab, Burlingame, CA, USA; 1:500</td>
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<tr>
<td>Monoclonal rat anti-mouse macrophage marker F4/80</td>
<td>Serotec; 1:50</td>
<td>Biotinylated anti-rat immunoglobulin</td>
<td>Vector Lab, Burlingame, CA, USA; 1:250</td>
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<tr>
<td>Polyclonal rabbit anti-mouse nitrotyrosine</td>
<td>Chemicon; 1:50</td>
<td>Biotinylated anti-rabbit immunoglobulin</td>
<td>Vector Lab, Burlingame, CA, USA; 1:500</td>
</tr>
<tr>
<td>Polyclonal goat anti-mouse gp91(\text{phox})</td>
<td>Santa Cruz; 1:100</td>
<td>Biotinylated anti-goat immunoglobulin</td>
<td>Vector Lab, Burlingame, CA, USA; 1:500</td>
</tr>
<tr>
<td>Polyclonal rabbit anti-mouse connective tissue growth factor (CTGF)</td>
<td>Abcam; 1:400</td>
<td>Biotinylated anti-rabbit immunoglobulin</td>
<td>Vector Lab, Burlingame, CA, USA; 1:500</td>
</tr>
</tbody>
</table>
**SUPPLEMENTAL Table 1 (continued):**

<table>
<thead>
<tr>
<th>Antibody/Reagent</th>
<th>Source</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polyclonal goat anti-mouse vascular endothelial growth factor (VEGF)</td>
<td>R&amp;D System;</td>
<td>1:50</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Biotinylated anti-goat immunoglobulin</td>
</tr>
<tr>
<td>Polyclonal rat anti-mouse vascular cell adhesion molecule-1 (VCAM-1)</td>
<td>BD</td>
<td>1:100</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Biotinylated rabbit anti-rat immunoglobulin</td>
</tr>
</tbody>
</table>

**SUPPLEMENTAL Table 2:** Primers and probes not described by us previously, and used in quantitative RT-PCR analysis are as follows.

<table>
<thead>
<tr>
<th>isoform</th>
<th>sense primer</th>
<th>anti-sense primer</th>
<th>probe</th>
</tr>
</thead>
<tbody>
<tr>
<td>VEGF</td>
<td>GCA CTG GAC CCT GGC TTT ACT</td>
<td>ATG GGA CTT CTG CTC TCC TTC TG</td>
<td>CTG TAC CTC CAC CAT GC</td>
</tr>
<tr>
<td>Sod1</td>
<td>TGT GAT CTC ACT CTC AGG AG</td>
<td>GGA CGG TGT GGC CAA TGT</td>
<td>CGG CCA ATG ATG GAA TGC</td>
</tr>
<tr>
<td>Sod2</td>
<td>CCT GAG CCC TAA GGG</td>
<td>GGG ACA TAT TAA TCA CAC CAT TTT CTG</td>
<td>CCC AAA GTC ACG CTT GAT AGC</td>
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<tr>
<td>gp91phox</td>
<td>CAA CTG GAC AGG AAC CT</td>
<td>AGT GCG TGT TGC TCG ACA AG</td>
<td>CCA AGC TAC CAT CTT ATG GAA AGT G</td>
</tr>
<tr>
<td>p65</td>
<td>AGC TCA AGA TCT GCC G</td>
<td>TCT CAC ATC CGA TTT TTG ATA ACC</td>
<td>CGA GGC AGC TCC CAG AGT T</td>
</tr>
<tr>
<td>RAGE</td>
<td>CAC AGC CCG GAT TG</td>
<td>GCT GTA GCT GGT GGT CAG AAC A</td>
<td>CCC CTT ACA GCT TAG CAC AAG TG</td>
</tr>
</tbody>
</table>
SUPPLEMENTAL TABLE 3: Characteristics of Mice at Conclusion of 20-week Study

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Non-diabetic animals</th>
<th>Diabetic animals</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ApoE⁻/⁻ (n=8)</td>
<td>ApoE⁻/⁻GPx1⁻/⁻ (n=8)</td>
</tr>
<tr>
<td>Body weight, g</td>
<td>27.9±0.6</td>
<td>31.7±0.3</td>
</tr>
<tr>
<td>Plasma glucose, mmol/L</td>
<td>9.2±0.8</td>
<td>10.3±1.0</td>
</tr>
<tr>
<td>Total cholesterol, mmol/L</td>
<td>9.8±1.4</td>
<td>13.1±1.5</td>
</tr>
<tr>
<td>Triglycerides, mmol/L</td>
<td>2.4±0.5</td>
<td>1.9±0.4</td>
</tr>
<tr>
<td>HDL, mmol/L</td>
<td>1.9±0.2</td>
<td>2.8±0.2</td>
</tr>
<tr>
<td>LDL, mmol/L</td>
<td>6.8±0.4</td>
<td>9.5±0.7</td>
</tr>
<tr>
<td>LDL/HDL ratio</td>
<td>3.7±0.2</td>
<td>3.5±0.1</td>
</tr>
</tbody>
</table>

*P<0.01, †P=0.06 vs non-diabetic counterpart. Data are shown as mean±S.E.M.

EXPANDED MATERIALS AND METHODS

Animal groups and experimental design

GPx1-deficient mice, back-crossed 10 times onto a C57Bl/J6 background and described by us previously,¹ were mated with C57Bl/J6 apolipoprotein E-deficient (ApoE⁻/⁻) mice to establish a line of ApoE⁻/⁻GPx1⁻/⁻ double knockout (dKO) mice. ApoE⁻/⁻ mice were generated through the breeding program and were maintained as a separate line. All studies were performed in male age-matched ApoE⁻/⁻ or ApoE⁻/⁻GPx1⁻/⁻ mice.

Eight-week old ApoE⁻/⁻ and ApoE⁻/⁻GPx1⁻/⁻ mice were rendered diabetic by two intraperitoneal injections of streptozotocin (STZ; Sigma-Aldrich, USA) on consecutive days, at a dose of 100mg/kg/day in 25mM citrate buffer (pH 4.2). ApoE⁻/⁻ and ApoE⁻/⁻GPx1⁻/⁻ mice, sham-injected with 25mM citrate buffer and studied concurrently, served as non-diabetic controls. Non-fasted blood glucose readings were measured every 3-4 weeks using a glucometer (Medisense Abbott Laboratories, CA, USA) after blood was obtained from the tail vein. Animals had unrestricted access to water and standard mouse chow. All studies were performed with approval of the Institutional Ethics Committee.

Animals were maintained for 20 weeks (20-week study) for enface aortic lesion analysis. In addition, to assess the status of other antioxidants and various pathways implicated in the progression of atherosclerosis, separate animal groups were assessed after 2 or 10 weeks of diabetes. Lesions were quantitated within the aortic sinus at both the 10 and 20-week time points. The study specifically involved enface assessment after 20-weeks of diabetes based on previous studies in our laboratory, where total plaque area was shown to be significantly increased in
diabetic ApoE−/− mice compared with non-diabetic controls. We specifically chose to investigate the expression of known pro-atherogenic markers after 10-weeks of diabetes in order to establish whether lack of GPx1 affected pro-atherogenic pathways at an earlier stage, thereby gaining valuable information about potential molecular pathways involved in the progression towards highly developed lesions. Since oxidative stress, and activation of redox-sensitive transcription factors, have been shown to occur within hours after glucose treatment and therefore as an early event of the diabetic milieu, we also felt that analysis at the earlier 2 week time-point would give us additional insight into the effects of a lack of GPx1 on other antioxidants of the antioxidant pathway.

Blood sampling and plasma biochemistry

Mice were anesthetized by an intraperitoneal injection of a 1.2% solution of 2,2,2-tribromoethanol (Sigma Chemical Co. USA) in citrate buffer (0.2mL/10g of body weight) after food had been withheld for 4 hours but with free access to water. The thoracic cavity was opened rapidly and 0.5-0.8 mL of blood drawn by direct puncture of the right ventricle. Blood samples were placed into heparinized tubes, centrifuged at 2,500 rpm and plasma frozen at –20°C until analyzed. Plasma glucose, cholesterol, high-density lipoprotein (HDL) and triacylglycerols were measured with commercial enzymatic kits using a Dimension RxL Chemistry Analyser (Dade Behring Diagnostics, Sydney, Australia). The Friedewald calculation was used to determine levels of low-density lipoprotein (LDL).

Tissue Collection

After blood sampling, hearts with aortas attached were rapidly removed, placed in cold saline, after which the aorta was severed close to the heart and cleaned of extraneous fat under a dissecting microscope. Cleaned aortas from the 20-week study were stored in neutral buffered formalin (10%) for assessment of atherosclerotic lesion area and subsequent immunohistochemical analysis. Cleaned aortas from the 2- and 10-week study were snap-frozen in liquid nitrogen and stored at –80°C for protein and gene expression studies respectively. Hearts, cut at a plane parallel to the tips of the atria, were fixed in 4% paraformaldehyde and frozen in OCT for subsequent lesion assessment within the sinus region.

Evaluation of Atherosclerotic Lesions

The extent and severity of atherosclerosis was assessed by two different approaches, firstly by the *en face* technique described by us previously to determine total and regional aortic plaque distribution and secondly by cross-sectional assessment through the aortic sinus. After *en face*
analysis, aortic tissue was embedded in paraffin and cross-sectional serial sections 3µm thick were prepared for immunohistochemical analysis.

Lesions within the aortic sinus region were assessed in 10µm cryostat-cut sections based on the method of Paigen et al.\textsuperscript{5} and described by us previously\textsuperscript{6}. Briefly, each mouse was perfused at a pressure of 80cm H\textsubscript{2}O with a washout solution (PBS) for 5 min followed by 4% paraformaldehyde in PBS (pH 7.0) 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. Hearts with the ascending aorta attached were removed under a dissecting microscope and cleaned of extraneous fat. The heart, with the ascending aorta attached, was cut at a plane parallel to the atrial appendages, washed three times in 30% sucrose (each of 15 hours’ duration), and embedded in O.C.T. compound (Tissue-Tek, Sakura Finetechical Co., Tokyo, Japan). Frozen cryostat sections were cut at 10 µm intervals from the left ventricular outflow tract through the aortic sinus. Beginning where the aortic leaflets first become visible, five 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 haematoxylin. The aortic sinus region was evaluated because this portion of the aorta is particularly susceptible to developing atherosclerotic lesions in mice fed HFD.\textsuperscript{7} Sections of the aortic sinus region were examined using light microscopy at 40X magnification under an Olympus BX50 optical microscope (Olympus Optical Co., Hamburg, Germany). Images were digitized using a JVC high-resolution camera and quantitated using Optimas (Media Cybernetics, Silver Spring, MD, version 6.2). For each mouse, lesion size/cross section was averaged to provide the mean lesion size/mouse. The results are expressed as lesion size (µm\textsuperscript{2}) ± S.E.M.

**Immunohistochemistry**

Serial aortic paraffin sections were stained for α-smooth muscle actin (α-SMA), the receptor for advanced glycated endproducts (RAGE), the macrophage marker F4/80, nitrotyrosine, gp91phox, connective tissue growth factor (CTGF) and vascular endothelial growth factor (VEGF). Frozen aortic sinus sections were stained for vascular cell adhesion molecule-1 (VCAM-1). Primary and secondary antibodies and dilutions are described in supplemental Table 1. Briefly, after hydration of sections, endogenous peroxidases were inactivated with 3% H\textsubscript{2}O\textsubscript{2}. Sections were then incubated with protein blocking agent prior to application of primary antibody overnight at 4°C, or 90min at room temperature for VCAM-1. This was followed by incubation with biotinylated secondary antibody for 10 min at room temperature. For both paraffin and frozen sections, endogenous non-specific binding for biotin was blocked using an avidin/biotin blocking kit (Vector Laboratories, Burlingame, CA) and both were incubated with Vectastain ABC reagent (Vector Laboratories). Peroxidase activity was identified by reaction with 3,3’-diaminobenzidine
tetrahydrochloride (Sigma Chemical Co.). Sections were counterstained with hematoxylin. Images were visualized under light microscopy (200X magnification; Olympus BX50, Olympus Optical), digitized with a JVC high-resolution camera and quantitated using Optimas (Media Cybernetics, Silver Spring, MD, version 6.2). Analysis was performed in a blinded fashion. On average 3 sections were assessed per mouse, and 7-9 mice analysed per group. Results were calculated as percentage positively stained tissue in the aortic media (for all proteins analysed, with the exception of VCAM-1 where the percentage positive staining was determined within the sinus region, at 40X magnification) and expressed relative to non-diabetic ApoE\(^{-/-}\) mice that were arbitrarily assigned a value of 1.

**Quantitative Reverse-Transcription Polymerase Chain Reaction (qRT-PCR)**

Total RNA extraction, preparation of DNA-free RNA and RNA reverse transcription have been described by us previously.\(^6\) Gene expression of VCAM-1, CTGF, VEGF, RAGE, \(\alpha\)-SMA, the NF-\(\kappa\)B subunit p65, and the antioxidant enzymes GPx1, GPx2, GPx3, GPx4, catalase, superoxide dismutase-1 (Sod1) and -2 (Sod2) and the NAD(P)H oxidase subunit gp91\(phox\), were analyzed by qRT-PCR as described by us previously.\(^6\) Most probes and primer sequences, purchased from Applied Biosystems (ABI, Foster City, CA, USA), have been described by us previously.\(^2,6\) Probes and primers for VEGF, Sod1, Sod2, gp91\(phox\), NF-\(\kappa\)B and RAGE are available in supplemental Table 2.

Briefly, total RNA was extracted after homogenisation of tissue (Polytron PT-MR2100; Kinematica AG) in TRIzol\(^R\) Reagent (Invitrogen Life Technologies). Contaminating DNA was removed after treatment with DNA-free\(^TM\) DNAse according to the manufacturer’s specifications (Ambion Inc, Austin, USA). 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). Gene expression was analysed by real-time quantitative RT-PCR using the Taqman system based on real-time detection of accumulated fluorescence (ABI Prism 7700, Perkin-Elmer Inc., Foster City, CA, USA). Fluorescence for each cycle was analyzed quantitatively by an ABI Prism 7700 Sequence Detection System (Perkin-Elmer, PE Biosystems). Gene expression was normalised relative to the expression of the housekeeping gene 18S ribosomal RNA (18S rRNA Taqman Control Reagent kit; ABI Prism 7700) that was multiplexed together with the gene of interest. Probes and primers were designed using a Primer Express program (ABI Prism 7700) with care taken to ensure that primers spanned an intron. Amplifications were performed with the following time course: 50\(^o\)C for 2 mins and 95\(^o\)C for 10 min and 40 cycles of 94\(^o\)C for 20s and at 60\(^o\)C for 1min. Each sample was tested in duplicate and results expressed relative to non-diabetic ApoE\(^{-/-}\) mice, which were arbitrarily assigned a value of 1.
Immunoblot analysis of individual aortas

Individual aortas, cleaned of extraneous fat, were homogenised in cold 20mM HEPES buffer, pH 7.2 containing 1mM EGTA, 210mM mannitol and 70mM sucrose. Cytosolic preparations were prepared by two-step centrifugation: 1,500g for 5 minutes at 4°C, followed by a 10,000g spin for 15 minutes at 4°C. The final supernatant was retained as the cytosolic protein preparation. Protein concentration was determined using a Pierce BCA Total Protein Assay Kit. Briefly, immunoblotting was performed as follows. In each lane, 20 µg (for Sod1 and catalase analysis) or 40µg (for GPx1 analysis) of cytosolic protein was electrophoresed in a 10% polyacrylamide gel under reducing conditions. Transfer onto BioTrace PVDF membrane (Pal Corp., Penscola, FL, USA) was performed using a semi-dry Trans-Blot SD (Bio-Rad) for 35 mins at 16V. Membranes were stored dry until immunodetection. We used rabbit polyclonal anti-GPx1 Ab (Abcam Ltd, Cambridge, UK, ab16798); rabbit polyclonal anti-Cu/Zn SOD Ab (Abcam Ltd, Cambridge, UK, ab13498); and rabbit polyclonal anti-catalase Ab (Abcam Ltd, Cambridge, UK, ab1877) as primary Ab’s. Pierce ImmunoPure Goat Anti-Rabbit IgG, Peroxidase conjugated, was used as secondary Ab. Mouse monoclonal anti-β-actin Ab (Abcam Ltd, Cambridge, UK, ab6276) and Goat Anti-Mouse IgG, Peroxidase conjugated (Dako), were used as primary and secondary Ab’s respectively to detect β-actin. Protein bands were detected through the use of an ECL kit (Pierce Supersignal West Pico Chemiluminescence Substrate) and visualised on a ChemiDoc XRS Documentation system (Bio-Rad Lab., Milan, Italy). A single band of 17kDa, 22kDa, and 65kDa corresponding to Sod1, GPx1 and catalase was detected with each antibody respectively. B-actin Ab detected a strong band at 42kDa. Band intensities were determined by densitometry. 4 aortas were analysed per group, and the results expressed as the mean ± S.E.M. Results are expressed relative to non-diabetic ApoE⁻/⁻ mice, which were arbitrarily assigned a value of 1.

REFERENCES:

