Glutathione Peroxidase-1 Deficiency Potentiates Dysregulatory Modifications of Endothelial Nitric Oxide Synthase and Vascular Dysfunction in Aging

Matthias Oelze,* Swenja Kröller-Schön,* Sebastian Steven, Edith Lubos, Christopher Doppler, Michael Hausding, Silke Tobias, Christoph Brochhausen, Huige Li, Michael Torzewski, Philip Wenzel, Markus Bachschmid, Karl J. Lackner, Eberhard Schulz, Thomas Münzel, Andreas Daiber

Abstract—Recently, we demonstrated that gene ablation of mitochondrial manganese superoxide dismutase and aldehyde dehydrogenase-2 markedly contributed to age-related vascular dysfunction and mitochondrial oxidative stress. The present study has sought to investigate the extent of vascular dysfunction and oxidant formation in glutathione peroxidase-1-deficient (GPx-1−/−) mice during the aging process with special emphasis on dysregulation (uncoupling) of the endothelial NO synthase. GPx-1−/− mice on a C57 black 6 (C57BL/6) background at 2, 6, and 12 months of age were used. Vascular function was significantly impaired in 12-month-old GPx-1−/− mice as compared with age-matched controls. Oxidant formation, detected by 3-nitrotyrosine staining and dihydroethidine-based fluorescence microtopography, was increased in the aged GPx-1−/− mice. Aging per se caused a substantial protein kinase C– and protein tyrosine kinase–dependent phosphorylation as well as S-glutathionylation of endothelial NO synthase associated with uncoupling, a phenomenon that was more pronounced in aged GPx-1−/− mice. GPx-1 ablation increased adhesion of leukocytes to cultured endothelial cells and CD68 and F4/80 staining in cardiac tissue. Aged GPx-1−/− mice displayed increased oxidant formation as compared with their wild-type littermates, triggering redox-signaling pathways associated with endothelial NO synthase dysfunction and uncoupling. Thus, our data demonstrate that aging leads to decreased NO bioavailability because of endothelial NO synthase dysfunction and uncoupling of the enzyme leading to endothelial dysfunction, vascular remodeling, and promotion of adhesion and infiltration of leukocytes into cardiovascular tissue, all of which was more prominent in aged GPx-1−/− mice. (Hypertension. 2014;63:390-396.) • Online Data Supplement

Key Words: aging • oxidative stress • vascular function

The incidence and frequency of cardiovascular diseases, such as hypertension, diabetes mellitus, and myocardial infarction, increase substantially with age.1 Increased oxidative stress from mitochondria and other enzymatic sources as well as vascular dysfunction manifest in aged animals.3 This observation points to a strong correlation between aging, oxidative stress, and, as a consequence, development of vascular/endothelial dysfunction.1 In 1954, Harman4 expressed for the first time the free radical theory of aging. The prevailing hypothesis is that an age-dependent increase in O2− will lead to more reactions with NO, subsequent ONOO− formation, and impaired vasorelaxation because of decreased vascular NO bioavailability.5

We have previously shown that genetic deletion of the mitochondrial antioxidant proteins manganese superoxide dismutase and aldehyde dehydrogenase-2 contributes to aging-dependent vascular dysfunction and mitochondrial oxidative stress.2 It is worth noting and of high clinical importance that antioxidant enzymes have a significant effect on the healthspan of animals during normal aging (e.g., indicated by less aging-associated cardiovascular complications during the sunset years) and also on the resistance to stress conditions.6

Previous studies have revealed increased mitochondrial and systemic oxidative stress in glutathione peroxidase-1 (GPx-1)-ablated mice7 and synergistic negative effects on vascular function in the setting of hyperlipidemia,8 diabetes mellitus,9 and hypertension.10 Moreover, increased senescence was demonstrated for fibroblasts from GPx-1−/− mice.7 Most importantly, a correlation between GPx-1 activity in blood cells and
Experimental Procedures

All the animals (160 male C57BL/6 or GPx-1−/− mice in total) were treated in accordance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health and the study was approved by the University Hospital Mainz Ethics Committee and the authorities (Landesuntersuchungsamt Rheinland-Pfalz, Germany). Isolation and use of human leukocytes was in accordance with the Helsinki Declaration and approved by the local ethics committee.

Additional list of materials, methodological details, and statistical analysis are described in the online-only Data Supplement.

Results

Effects of Aging and GPx-1 Deficiency on Vascular Function and Cardiovascular Oxidative Stress

Vascular function was assessed by ACh-, nitroglycerin (GTN)-, and diethylamine NONOate–dependent relaxation in isolated aortic ring segments and was moderately impaired by the aging process in wild-type mice (Figure 1A–1C) in contrast to severe endothelial dysfunction in GPx-1−/− mice. The tetrahydrobiopterin (BH4) precursor sepiapterin normalized endothelial function, indicating a role for uncoupled eNOS for endothelial dysfunction (Figure S1 in the online-only Data Supplement). The sensitivity to the vasoconstrictor PGF2α in old wild-type and GPx-1−/− mice was increased (Figure S2A). We also demonstrated impaired vasodilatory effects of GTN and the NO donor, diethylamine NONOate, in accordance with the finding that aged GPx-1−/− but not wild-type mice revealed a trend toward decreased expression of soluble guanylyl cyclase subunits α and β, also reflected by the trend that phosphorylated vasodilator-stimulated phosphoprotein (VASP) at Ser239 (p-VASP) was decreased in both animal strains (Figure S2B). The expression of cyclic guanosine monophosphate–dependent protein kinase-1 was not significantly changed and the ratio of Ser239 phosphorylated VASP and total VASP showed a similar tendency as shown by p-VASP data (Figure S3).

Vasodilation to the adenylyl cyclase (cyclic adenosine monophosphate dependent) activator iloprost was significantly reduced in old GPx-1−/− mice (Figure S4) as compared with young wild-type and GPx-1−/− mice. Together with the observed reduction of GTN- and NONOate-induced vasodilation, these observations suggest an impaired capacity of the vessel to dilate. Vascular remodeling such as fibrosis of the intima and subsequent vascular stiffening may explain this observation (Figures S5 and S6 and Tables S1 and S2).

Vascular RONS formation was significantly increased with advancing age in both B6 control and GPx-1−/− mice, but overall levels were significantly higher in GPx-1−/− mice across all age groups (2, 6, and 12 months; Figure 2A). Mitochondrial RONS production (mitoSOX staining) was increased age-dependently in aortae of B6 control animals, but plateaued in the 6- to 12-month-old GPx-1−/− mice (Figure 2B). A similar trend was observed with isolated membrane fraction and lucigenin enhanced chemiluminescence as a readout of cardiac NADPH oxidase activity as well as with isolated cardiac mitochondria and L-012–enhanced chemiluminescence (Figure S7). eNOS uncoupling as assessed by endothelial dihydroethidium (DHE) staining was more pronounced in GPx-1−/− than in wild-type mice throughout all groups of age and showed an age-dependent increase (Figure S8). Endothelial DHE staining was suppressed by sepiapterin, l-NAME, and VAS2870 (Figure S8). The immunohistochemical staining for phagocytic NADPH oxidase

Figure 1. Glutathione peroxidase-1 (GPx-1) deficiency aggravates vascular dysfunction during the aging process. Aortic relaxation was determined by isometric tension recording in response to endothelium-dependent (ACh; A), endothelium-independent with requirement of bioactivation (GTN; B), and endothelium-independent without requirement of bioactivation (diethylamine NONOate; C) from wild-type and GPx-1–deficient mice at different age. Data are means±SEM of 10 to 16 aortic ring segments from 6 to 9 animals per group (A-D). #P<0.05 vs GPx-1−/− (2 mo); $P<0.05 vs GPx-1−/− (6 mo); §P<0.05 vs GPx-1−/− (12 mo); and §§P<0.05 vs B6 Ctr (2 mo). Statistical analysis was performed by 2-way ANOVA (A–C); for the sake of clarity, only 1 significance symbol is shown for the entire curves (A–C).
(Nox2) was clearly restricted to the adventitial cell layer in both mouse strains (Figure S9). Oxidative stress was also measured in aorta and heart by 3-nitrotyrosine– and 4-hydroxynonenal–modified proteins (Figure 2C; Figure S10). Tyrosine nitration, a marker for in vivo peroxynitrite formation, increased in all age groups and by trend (aorta) or significantly (heart) stronger in old GPx-1−/− mice. Levels of 4-hydroxynonenal, a marker for lipid peroxidation, were significantly increased in all age groups and by trend (aorta) or significantly (heart) stronger in old GPx-1−/− mice. The increased carbonyl stress in aorta from old GPx-1−/− mice was also confirmed by oxy blot analysis (Figure S11). The increases in nitration in old animals could also be confirmed using immunohistochemical staining procedures (Figure 2D).

Qualitatively, nitrotyrosine staining in GPx-1−/− aorta was found in the media, adventitial cell layers, and intima, whereas in wild-type aorta, it was rather restricted to the intima.

Age and GPx-1−/−-Dependent Dysregulation of eNOS

Phosphorylation of eNOS at Thr495 by protein kinase C represents an inactivation and uncoupling pathway for eNOS.12 Phosphorylation of eNOS at Thr495 in heart tissue from GPx-1−/− mice increased with age (Figure 3A). eNOS S-glutathionylation leads to dysfunction and uncoupling of the enzyme.13 eNOS S-glutathionylation was augmented in an age-dependent fashion (Figure 3B) and showed a 2-fold increase.

Figure 2. Glutathione peroxidase-1 (GPx-1) deficiency moderately aggravates vascular oxidative stress during the aging process. Vascular oxidative stress was assessed by dihydroethidine-dependent fluorescence microtopography in aortic cryosections (A). For determination of mitochondrial vascular reactive oxygen and nitrogen species formation, the mitochondria-targeted DHE analog mitoSOX was used (B). Representative microscope images are shown below the densitometric quantifications (more images are available in Figure S16). Cardiac oxidative stress was assessed by dot blot analysis for 3-nitrotyrosine– and 4-hydroxynonenal–positive proteins by using specific antibodies (C). Representative dot blots are shown at the bottom of each densitometric quantification graph. D, Immunohistochemical staining for vascular 3-nitrotyrosine–positive proteins in paraffinized aortic sections. Magnification, ×40; E indicates endothelium. Representative for ≥3 animals per group. Data are mean±SEM of 16 to 24 cryosections from 6 to 9 animals per group (A and B) and protein samples from 6 (C) animals per group. *P<0.05 vs 2 mo group; #P<0.05 vs 6 mo group; $, §: P<0.05 vs respective age-matched B6 Ctr group.
increase in young GPx-1–/– mice and ≈2.5-fold increase in old GPx-1–/– mice as compared with their age-matched wild-type littermates (Figure 3C). A similar pattern was observed for aortic eNOS S-glutathionylation and Thr495 phosphorylation in young and old GPx-1–/– mice (Figure 3D). Phosphorylation of eNOS at Tyr657 by protein tyrosine kinase 2 renders eNOS dysfunctional.14 Heart tissue from GPx-1–/– mice showed augmented phosphorylation of Tyr657 on eNOS with increasing age with ≈3-fold increase in old GPx-1–/– mice compared with wild type (Figure S12). However, it remains to be known whether the described eNOS modifications are markers or makers of eNOS uncoupling/dysfunction because we do not know their extent on a molecular basis (eg, how much S-glutathionylation or phosphorylation per eNOS molecule).

**GPX-1 Deficiency in Cultured Endothelial Cells Increases Leukocyte Adhesion**

Effects of GPx-1 silencing on leukocyte adhesion to cultured endothelial cells (EA.hy926) were measured by the expression of the p67phox cytosolic subunit of phagocytic NADPH oxidase (Figure 4A and 4B) or by phorbol ester-stimulated oxidative burst (Figure S13) from adherent leukocytes after 2 washing steps. The number of adherent leukocytes was increased in GPx-1–silenced endothelial cells. As an in vivo correlate of this inflammatory phenotype of GPx-1–silenced cells, aortic or cardiac tissue lysates obtained from old GPx-1–/– mice were positive for CD68 and F4/80. These markers for monocytes/macrophages indicated increased vascular leukocyte infiltration in old GPx-1–/– mice (Figure 4C and 4D). A role of inflammatory cells for the observed oxidative damage (nitration), collagen deposition, and vascular dysfunction was supported by the increased adventitial ROS formation in old animals (Figure S14).

**Discussion**

In the present study, we demonstrate, for the first time to our knowledge, that aging per se leads to eNOS dysfunction and eNOS uncoupling via increased adverse phosphorylation and...
S-glutathionylation of the enzyme. We also established that GPx-1 deficiency resulted in a phenotype of endothelial and vascular dysfunction that was substantially potentiated by the aging process. By using oxidative stress–prone GPx-1−/− mice (a model representing decreased breakdown of cellular hydrogen peroxide) in a study of the aging process, we can now provide stronger mechanistic links between oxidative stress, eNOS dysfunction, and vascular dysfunction in aging animals. Most importantly, a correlation between GPx-1 activity in blood cells and cardiovascular risk was previously reported.11 In the present study, the striking endothelial dysfunction observed in aged GPx-1−/− mice could be partly explained by adverse phosphorylation and S-glutathionylation of eNOS.

eNOS phosphorylation at Thr495 (protein kinase C dependent) and Tyr657 (tyrosine kinase 2 dependent) has been described to cause both enzymatic dysfunction (Thr495 and Tyr657) and uncoupling (only confirmed for Thr495 to date) of the enzyme, respectively,12,14 and it is important to mention that both kinases are activated by hydrogen peroxide, superoxide, and peroxynitrite.14,15 Tyr657 phosphorylation, according to this report, functions as a master switch for eNOS activity by interfering with the electron flow in the reductase domain and inhibiting the NO-synthesizing activity of eNOS.14 In another report, the same group showed that the Tyr657 phosphomimetic mutants of eNOS—Y657D and Y657E—showed diminished NO formation without increased superoxide formation, which would argue

---

**Figure 4.** Glutathione peroxidase-1 (GPx-1) deficiency aggravates adhesion of human leukocytes to cultured human endothelial cells and induces a proinflammatory phenotype during the aging process. EA.hy926 cells (Ctr, siCtr, and siGPx-1) were incubated with PMN (1×10^6 cells/mL; A) or monocytes/lymphocytes (1×10^6 cells/mL; B) and LPS (10 μg/mL), termed as low conditions. Adherent PMN were quantified on lysis by the content of p67phox protein. C and D, Aortic CD68 and cardiac F4/80 protein expression as a readout of cardiovascular monocyte/macrophage infiltration was determined by dot blot analysis. Representative blots are shown below the densitometric quantification. Data are means±SEM of ≥3 (A–D) protein expression assays pooled from ≥4 different wells and 3 (C) or 5 to 6 (D) protein expression assays each pooled from ≥2 animals per group. *P<0.05 vs respective young (2 mo) group; §P<0.05 vs GPx-1−/− (12 mo). A and B, All tested groups were significantly different against the other groups.
against uncoupling of the enzyme by this modification.\textsuperscript{16} We also detected S-glutathionylation of the eNOS reductase domain, causing eNOS dysfunction and uncoupling,\textsuperscript{13,17,18} for the first time, in the aging vasculature, which was most pronounced in aged \textit{GPx-1}\textsuperscript{−/−} mice. Therefore, adverse phosphorylation pattern and S-glutathionylation processes, but also oxidative depletion of BH\textsubscript{4} or RONS-induced augmentation of the plasma levels of asymmetrical dimethylarginine, cause the so-called redox switch within eNOS that may be mediated by the increased peroxynitrite formation observed, in particular, in GPx-1 deficiency per se or the aging vasculature, in general.\textsuperscript{19,20} The protective effects of sepiapterin observed here support a role of BH\textsubscript{4} deficiency for eNOS uncoupling and dysfunction in aged animals, especially in GPx-1 deficiency. Prevention of eNOS uncoupling by Nox inhibition is compatible with the suppression of oxidative depletion of BH\textsubscript{4} but also of protein kinase C and protein tyrosine kinase 2 activation and of eNOS S-glutathionylation as recently shown for p47\textsubscript{phox} and gp91\textsubscript{phox} deficiency.\textsuperscript{21} The cellular redox state of sepiapterin observed here supports a role of BH\textsubscript{4} deficiency for eNOS uncoupling and dysfunction in aged animals, especially in GPx-1 deficiency. Prevention of eNOS uncoupling by Nox inhibition is compatible with the suppression of oxidative depletion of BH\textsubscript{4} but also of protein kinase C and protein tyrosine kinase 2 activation and of eNOS S-glutathionylation as recently shown for p47\textsubscript{phox} and gp91\textsubscript{phox} deficiency.\textsuperscript{21} The cellular redox state (GSH/GSSG ratio) could also directly affect S-glutathionylation of enzymes and, therefore, contribute to the coupled state of eNOS (see online-only Data Supplement). In 2006, Smith et al\textsuperscript{23} proposed that the decline in endothelial GSH during the aging process may contribute to a change of eNOS phosphorylation pattern (decline in P-Ser1176 and increase in P-Thr494) that was associated with a loss of vascular NO bioavailability, increased proinflammatory cytokines, and impaired endothelium-dependent vasodilation. This hypothesis is compatible with the results presented here on a decline in cellular-reduced thiols by trend in the old \textit{GPx-1}\textsuperscript{−/−} mice (Figure S15), eNOS dysfunction by adverse phosphorylation, and S-glutathionylation and vascular inflammation in these animals.

We also observed substantial desensitization of the vasculature to the endothelium-independent vasodilators GTN and diethylamine NONOate in aged \textit{GPx-1}\textsuperscript{−/−} mice. There may be several explanations for this phenomenon. It is known that, for example, in the setting of hypercholesterolemia, the capacity of NO to lower intracellular calcium concentrations (thereby causing relaxation) is greatly reduced.\textsuperscript{23} In addition, remodeling (increase in media thickness) may, at least partially, contribute to the impairment of the endothelium-independent vasodilation in the aged mice. The direct oxidative breakdown of NO by increased ROS formation in the smooth muscle cell layer may represent another reason for endothelial/vascular dysfunction because vascular superoxide was largely increased in old \textit{GPx-1}\textsuperscript{−/−} deficient mice. Finally, it is known that superoxide as well as peroxynitrite is able to inhibit the activation of the soluble guanylyl cyclase.\textsuperscript{24}

We also measured a significant increase in RONS (by using DHE and 3-nitrotyrosine staining) in old \textit{GPx-1}\textsuperscript{−/−} mice as compared with their age-matched controls. Previous reports have shown an inflammatory phenotype in \textit{GPx-1}\textsuperscript{−/−} silenced cultured endothelial cells associated with increased adhesion molecule and cytokine expression involving the JNK/ERK pathway.\textsuperscript{25} Here, we demonstrate increased adhesion of isolated human leukocytes to cultured \textit{GPx-1}\textsuperscript{−/−} silenced endothelial cells and an increase in CD68 and F4/80 protein levels, markers for macrophages/monocytes, in aortic tissue of aged mice. Adventitial accumulation of these cells would be also compatible with the observed increase in ROS formation (DHE staining), collagen content, and 3-nitrotyrosine/Nox2 staining in the adventitia. This apparently age-dependent increase in inflammation of cardiovascular tissue with leukocytes was more pronounced in \textit{GPx-1}\textsuperscript{−/−} deficient mice, suggesting that \textit{GPx-1} deficiency may lead to an inflammatory phenotype of the vasculature, a condition that was previously reported to contribute to increased oxidative stress and vascular/endothelial dysfunction.\textsuperscript{26}

A limitation of the study is that most RONS measurements are based on dihydroethidine-dependent fluorescence microtopography, a technique that was previously questioned for its specificity and reflection of intact cellular function.\textsuperscript{27} In defense of this technique, we recently reported that L-NAME but not d-NAME blocks endothelial DHE staining derived from uncoupled eNOS,\textsuperscript{21} and we show here that NADPH oxidase inhibition and sepiapterin preincubation suppresses endothelial DHE signal.

\section*{Perspectives}

Here, we demonstrate that \textit{GPx-1} ablation in aging animals has a substantial impact on the burden of oxidative stress and injury, contributing to the observed difference between vascular dysfunction in aged wild-type and \textit{GPx-1}\textsuperscript{−/−} mice. This observation goes with previous reports on an aggravation of oxidative vascular damage in atherosclerotic \textit{GPx-1}\textsuperscript{−/−} \textit{ApoE}\textsuperscript{−/−} mice\textsuperscript{28} and with the finding that in patients with coronary artery disease a low level of activity of red blood cell GPx-1 is independently associated with an increased risk of cardiovascular events.\textsuperscript{11,29} Increasing \textit{GPx-1} activity, for example, by selenium supplementation might lower the risk of cardiovascular events, which may be explained, at least in part, by the prevention of eNOS dysfunction in the aging vasculature.\textsuperscript{30}

\section*{Acknowledgments}

We thank Jörg Schreiner, Angelica Karpi, Nicole Glas, Jessica Rudolph, and Bettina Mros for their expert technical assistance. This article contains results that are part of the doctoral thesis of C.D.

\section*{Sources of Funding}

The present work was supported by generous financial support by the Stiftung Mainzer Herz (to M.O., T.M., and A.D) and by funds from the Federal Ministry of Education and Research (BMBF 01EO1003, to P.W., T.M., and A.D).

\section*{Disclosures}

None.

\section*{References}


---

**Novelty and Significance**

**What Is New?**

- Glutathione peroxidase-1 (Gpx1) deficiency synergistically increases endothelial and vascular dysfunction and creates a proinflammatory phenotype with increasing age.

- These effects are mediated by increased oxidative stress and related dysregulation of the endothelial NO synthase (eNOS) associated with S-glutathionylation and adverse phosphorylation.

**What Is Relevant?**

- Gpx1 expression and activity as well as endothelial function and oxidative stress are directly correlated with cardiovascular events in patients with coronary artery disease.

- eNOS S-glutathionylation is a new marker for eNOS dysfunction and cardiovascular complications.

**Summary**

The finding that Gpx1 deficiency leads to dysregulation of eNOS and creates an activated endothelium in aged mice provides the mechanistic basis for impaired endothelial function and increased cardiovascular events with increasing age. The involvement of Gpx1 as the most abundant isoform of glutathione peroxidases and oxidative stress suggests new antioxidant therapeutic approaches to counteract the deleterious effects of the aging process on the cardiovascular function.
Glutathione Peroxidase-1 Deficiency Potentiates Dysregulatory Modifications of Endothelial Nitric Oxide Synthase and Vascular Dysfunction in Aging
Matthias Oelze, Swenja Kröller-Schön, Sebastian Steven, Edith Lubos, Christopher Doppler, Michael Hausding, Silke Tobias, Christoph Brochhausen, Huige Li, Michael Torzewski, Philip Wenzel, Markus Bachschmid, Karl J. Lackner, Eberhard Schulz, Thomas Münzel and Andreas Daiber

Hypertension. 2014;63:390-396; originally published online December 2, 2013; doi: 10.1161/HYPERTENSIONAHA.113.01602

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://hyper.ahajournals.org/content/63/2/390

Data Supplement (unedited) at:
http://hyper.ahajournals.org/content/suppl/2013/12/02/HYPERTENSIONAHA.113.01602.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Hypertension can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Hypertension is online at:
http://hyper.ahajournals.org//subscriptions/
Online supplement

Glutathione Peroxidase-1-Deficiency Potentiates Dysregulatory Modifications of Endothelial Nitric Oxide Synthase and Vascular Dysfunction in Aging

Matthias Oelze*, Swenja Kröller-Schön*, Sebastian Steven, Edith Lubos, Christopher Doppler, Michael Hausding, Silke Tobias, Christoph Brochhausen, Huige Li, Michael Torzewski, Philip Wenzel, Markus Bachschmid, Karl J. Lackner, Eberhard Schulz, Thomas Münzel, and Andreas Daiber

From the 2nd Medical Clinic, Department of Cardiology (M.O., S.K.-S., S.S., C.D. M.H., P.W., E.S., T.M., A.D.), Center of Thrombosis and Hemostasis (M.H., P.W.), Department of Pharmacology (S.T., H.L.), Institute of Pathology (C.B.), and Institute of Clinical Chemistry and Laboratory Medicine (K.J.L.), Medical Center of the Johannes Gutenberg University, Mainz, Germany; Department of Laboratory Medicine, Robert-Bosch-Hospital, Stuttgart, Germany (M.T.); Department of General and Interventional Cardiology, University Heart Center Hamburg, University Medical Center Hamburg-Eppendorf, Hamburg, Germany (E.L.); The Boston University Medical Center, Boston, USA (M.B.).
Extended Methods

Materials

For isometric tension studies, nitroglycerin (GTN) was used from a Nitrolingual infusion solution (1 mg/ml) from G.Pohl-Boskamp (Hohenlockstedt, Germany). Diethyamine NONOate (DEA/NO) was from Cayman Chemicals (Ann Arbor, MI, USA). Lipopolysaccharide (LPS, Sal.Typh.) was from Sigma-Aldrich (Munich, Germany). L-012 (8-amino-5-chloro-7-phenylpyrido[3,4-d]pyridazine-1,4-(2H,3H)dione sodium salt) was purchased from Wako Pure Chemical Industries (Osaka, Japan). All other chemicals were of analytical grade and were obtained from Sigma-Aldrich, Fluka or Merck.

Animal Model and Human Material

All of the animals (160 male mice in total) were treated in accordance with the Guide for the Care and Use of Laboratory Animals as adopted by the National Institutes of Health and were granted by the University Hospital Mainz Ethics Committee and the authorities (Landesuntersuchungsamt Rheinland-Pfalz, Germany). GPx-1−/− and C57BL/6 mice were bred in the central animal facility of the University Medical Center in Mainz and were given ad libitum access to water and standard food. Mice were used in 3 different age groups: 2, 6 and 12 months. Heart and aorta were removed under isoflurane anesthesia as previously described. Isolation and use of human leukocytes was in accordance with the Helsinki Declaration and approved by the local ethical committee.

Vascular Function

Vasodilation to endothelial-dependent (acetylcholine (ACh)) and −independent (nitroglycerin (GTN) and diethyamine NONOate (DEANO)) vasodilators was assessed by isometric tension recordings in prostaglandin F2α (PGF2α) preconstricted isolated aortic ring segments as previously described. The sensitivity of the aortae for the constrictor PGF2α (2 µM) was tested directly after determination of the maximal vasoconstriction in response to 80 mM KCl and prior to the first relaxation-concentration-curve. For some tension experiments the aortic ring segments were preincubated for 1 h with the BH4 precursor sepiapterin (100 µM) and PEG-SOD (200 U/ml) to prevent its autoxidation, prior to the tension recording.

Vascular and Cardiac Formation of RONS

Vascular RONS formation was determined by dihydroethidine or mitochondria-targeted dihydroethidine (mitoSOX, 1 µM)-dependent fluorescence microtopography in aortic cryosections. Endothelial DHE staining was quantified by densitometric analysis of the endothelial cell layer. Some cryo sections were incubated for 30 min with sepiapterin (100 µM), the Nox inhibitor VAS2870 (20 µM) or the NOS inhibitor L-NAME (500 µM) in order to test for eNOS uncoupling and NADPH oxidase derived ROS. Adventitial DHE staining was quantified by densitometric analysis of the adventitial cell layer.

Cardiac oxidative stress was assessed by enhanced chemiluminescence (ECL) in isolated cardiac mitochondrial and membranous fractions. Isolated mitochondria were prepared from rat hearts according to a previously published protocol and ROS formation was detected by L-012 (100 µM) ECL as recently described. Mitochondrial suspensions were diluted to a final protein concentration of 0.1mg/ml in 0.5ml of PBS buffer containing L-012 (100 µM). ROS production was detected after stimulation with succinate (5mM final concentration). The ECL was registered at intervals of 30s over 5min with a Lumat chemiluminometer (Berthold Techn., Bad Wildbad, Germany) and the signal was expressed as counts/min at 5min. Membrane fractions were prepared and NADPH oxidase activity was measured by lucigenin (5 µM) ECL in the presence of NADPH (200 µM) according to a published protocol.
Cardiac oxidative stress was also assessed by dot blot analysis of cardiac tissues, which was modified from a previous report. Protein tyrosine nitration was detected using a specific antibody for 3-nitrotyrosine (3NT). Increased lipid peroxidation was monitored using a specific antibody for 4-hydroxynonenal (4HNE)-positive proteins. Briefly, 100 µl (0.2 µg/µl protein based on Bradford analysis) of the homogenized tissue sample was transferred to a Protran BA85 (0.45µm) nitrocellulose membrane (Schleicher&Schuell, Dassel, Germany) by a Minifold I vacuum Dot-Blot system (Schleicher&Schuell, Dassel, Germany). Each slot was washed with 250µl PBS and the membrane was dried for 15min at 60°C. For detection of nitrated protein, a mouse monoclonal 3NT antibody (Upstate Biotechnology, MA, USA) was used at a dilution of 1:1000. 4HNE-positive protein was detected using a mouse monoclonal 4HNE antibody (Percipio Biosciences, Foster City, CA, USA) was used at a dilution of 1:1000. Positive bands were detected by enhanced chemiluminescence after incubation with a peroxygenase-coupled secondary antibody (GAM-POX, 1:5000) (Vector Laboratories, CA, USA). Protein carbonyl groups were detected with the oxy blot protocol by dot blot analysis. Briefly, the membrane was incubated with 2,4-dinitrophenylhydrazine (100 µg/ml) for 5 min (not less and not more!) in order to convert all non-peptide carbonyl groups to 2,4-dinitrophenylhydrazones (DNPH), which were then stained with a specific rabbit polyclonal antibody against DNPH-protein adducts (Serotec, Oxford, UK) was used at a dilution of 1:1000. All incubation and washing steps were performed according to the manufacturer’s instructions. Densitometric quantification of the dots was performed as described in the immunoprecipitation and Western blot section.

Phosphorylation and S-glutathionylation of Endothelial Nitric Oxide Synthase in the Heart

Immunoprecipitation of eNOS and subsequent immunoblotting of the precipitate for S-glutathionylation, and phosphorylation at Thr495 and Tyr657 was performed according to a standard protocol as recently published. M-280 sheep anti-mouse IgG coated beads from Invitrogen (Darmstadt, Germany) were used along with a monoclonal mouse eNOS (Biosciences, USA) antibody. The beads were loaded with the eNOS antibody and cross-linked according to the manufacturer’s instructions. Next, cardiac (and in one experiment aortic) homogenates were incubated with the eNOS antibody beads, precipitated with a magnet, washed and transferred to the gel and subjected to SDS-PAGE followed by a standard Western blot procedure using a monoclonal mouse antibody against S-glutathionylated proteins from Virogen (Watertown, MA, USA) at a dilution of 1:1000 under non-reducing conditions. Disappearance of the signal upon incubation with 2-mercaptoethanol served as a control. After stripping of the membrane, the bands were immunoblotted for eNOS to allow normalization of the signals. A similar immunoprecipitation procedure was used to detect the protein kinase C-dependent phosphorylation site of eNOS, Thr495, and the protein tyrosine kinase-dependent phosphorylation site of eNOS, Tyr657. Western blotting was performed using a specific polyclonal rabbit antibody against phospho-Thr495-eNOS (1:2000, Upstate Biotechnology, Billerica, MA, USA) or a mixture of mouse monoclonal antibodies against phospho-Tyr (each 1:1000;P-Tyr 100, Cell Signaling; Py20, Santa Cruz; Py20, BD Bioscience; clone 4G10, Millipore). After stripping of the membrane, the bands were immunoblotted for eNOS to allow normalization of the signals. Detection and quantification were performed by enhanced chemiluminescence (ECL) with peroxidase conjugated anti-rabbit/mouse (1:10000, Vector Lab., Burlingame, CA) secondary antibodies. Densitometric quantification of antibody-specific bands was performed with a ChemiLux Imager (CsX-1400M, Intas, Göttingen, Germany) and Gel-Pro Analyzer software (Media Cybernetics, Bethesda, MD).

Western Blotting and Dot Blot Analysis of Other Proteins
The procedures were similar to those described above but aortic tissue was used in all Western blot experiments. Protein samples were analyzed by Western blot analysis for sGCα1 and sGCβ1 (rabbit polyclonal, 1:10000 and 1:500, Abcam, Cambridge, UK), phospho-Ser239-VASP (mouse monoclonal, 1.5µg/ml, Millipore, Billierica, MA, USA), VASP (rabbit polyclonal, 1:2500, immunoGlobe, Himmelstadt, Germany), cGK-1 (goat polyclonal, 1:200, SantaCruz, Dallas, USA) and monoclonal mouse α-actinin (1:2500, Sigma-Aldrich). For dot blot analysis regarding content of inflammatory cells in cardiac tissue we used a CD68 antibody (monoclonal mouse, 1:500, Abcam) and a F4/80 antibody (rat monoclonal, 1:250, eBioscience, San Diego, CA, USA).

Leukocyte Adhesion to Cultured Endothelial Cells

The effect of GPx-1 deficiency was also tested by quantification of human leukocyte adhesion to cultured human endothelial cells (EA.hy926 cells [immortal human endothelial hybrid cell line], a kind gift of C.J. Edgell, University of North Carolina at Chapel Hill, USA) by different methods. The culture conditions were previously published 14. The silencing of GPx-1 by siRNA in human endothelial cells was recently published 15 and the successful suppression of GPx-1 at the protein level was tested using a specific polyclonal rabbit antibody (1µg/ml, Abcam, Cambridge, UK). After incubation of the confluent endothelial cells in 6- or 96-well plates with isolated neutrophils (PMN) or isolated monocytes/lymphocytes (WBC) (1x10^5 and 5x10^5 cells/ml) together with LPS (5 and 25 µg/ml) for 30 min, the endothelial cells were washed twice with warm PBS. Adherent PMN were quantified by phorbol ester dibutyrate (PDBu, 1 µM)-triggered ROS formation (measured by L-012 (100 µM) ECL) after 30 min in 96-well plates. Adherent PMN were also quantified by p67phox expression (a constituent of the phagocytic NADPH oxidase not expressed in EA.hy 926 cells). The washed cells from two wells (from 6-well plates) were pooled in Laemmli buffer and subjected to SDS-PAGE and Western blotting using a mouse monoclonal p67phox antibody (1:500) from Transduction Laboratories (Lexington, USA) and a polyclonal rabbit β-actin antibody (1:2500, Sigma-Aldrich, Munich, Germany) as the loading control 16. Detection was performed by ECL with peroxidase conjugated anti–rabbit/mouse (1:10000, Vector Lab., Burlingame, USA) secondary antibodies. The antibody-specific bands were quantified by densitometry as described in the immunoprecipitation and Western blot section.

Iloprost-dependent Relaxation, Histology and Immunohistochemistry

The NO/cGMP independent vasodilation was assessed by isometric tension recordings in response to the stable prostacyclin analogue Iloprost in prostaglandin F2α (PGF2α) preconstricted isolated aortic ring segments as previously described 17, 18. Iloprost should induce vasodilation via the adenylyl cyclase, cAMP and cAMP-dependent kinase pathway. The sensitivity of the aortae for Iloprost upon PGF2α (2 µM) preconstriction was tested directly after determination of the maximal vasoconstriction in response to 80 mM KCl and prior to the first relaxation-concentration-curve.

The immunohistochemical protocols were published previously 19, 20. Briefly, aortic segments were fixed in parafomaldehyde (4%) and paraffin-embedded. For unmasking of the antigen the samples were treated in a steamer with target retrieval solution (pH 6 or 9 depending on the antibody). Sections were stained for nitrotyrosine using a rabbit antibody (Millipore, Germany) diluted 1:100 in 2.5% horse normal serum (Vector laboratories, Burlingame, CA, USA) and for NOX-2 (gp91phox) using a mouse antibody (BD Transduction laboratories, Germany) diluted 1:100 in diluent (M.O.M.–Kit, Vector laboratories). Following the species of primary antibody, appropriate biotinylated secondary antibodies were used after dilution following the manufacturer’s instructions. For immunochemical detection ABC
reagent (Vector laboratories) and then DAB reagent (Peroxidase substrate Kit, Vector laboratories) were used as substrates.

The histological protocols were published previously. Sirius red staining for vascular fibrosis was performed with paraffin-embedded samples of aortic tissue upon de-paraffination. Afterwards the nuclei were pre-stained with hemalum. Then samples were stained for 1 hour in 0.1% with Sirius red solution containing saturated picric acid (1.2%). Finally, tissue samples were dehydrated with 70%, 96% and 100% isopropanol and coverslipped with Entellan®. Trichromstaining (according to Goldner et al.) was performed with paraffin-embedded samples of aortic tissue upon de-paraffination. Afterwards the nuclei were pre-stained with haematoxylin (according to Harris et al.). Then samples were stained for 5 minutes with mallow red containing 100% acetic acid, fuchsin acid and Orange G (Merck, Darmstadt, Germany), then for 15 minutes in 1% molybdatophosphoric acid hydrate (VWR, Darmstadt, Germany), then for 5 minutes in acid light green. Finally tissue samples were dehydrated in glacial acetic acid and 100% ethanol and coverslipped in Entellan®.

Determination of Cellular Reduced Thiol Levels
Cardiac tissue reduced thiol content (protein-bound and low molecular weight) was measured by Elman’s reagent (5,5’-dithiobis-(2-nitrobenzoic acid), DTNB) dependent photometry (absorbance change at 430 nm). Briefly, hearts were homogenized in 1 ml of a 1 mM 5,5’-dithiobis-(2-nitrobenzoic acid) (DTNB) solution, diluted 1:40 in DTNB (0.5 mM) and after 5 min the difference in absorbance at 430 nm was measured. The values were normalized to the wet weight of the hearts. Non-protein reduced thiols (NPT) were determined by HPLC-based quantification of DTNB-cysteine, DTNB-GSH adducts as well as free nitrothiobenzoate (NTB). Briefly, hearts were homogenized in 1 ml Krebs-Hepes buffer and centrifuged for 10 min at 4 °C and 20,000g. The supernatant was filtered by size exclusion filtration through a Microcon filter device (10 kDa) from Millipore Co. (Billerica, MA, USA) and the eluate was incubated for 5 min with DTNB (2 mM). 50 µl of these samples were subjected to HPLC analysis on a Jasco HPLC system equipped with an autosampler and a C18-Nucleosil 100-3 (125x4 mm) reversed phase column (Macherey & Nagel, Düren, Germany). The mobile phase consisted of component (A) citric acid buffer (50 mM, pH 2) and component (B) acetonitrile/water (90:10 v/v%) and a gradient was applied: 0 min, 0 % B; 9 min, 100 % B; 10 min 100 % B; 10.5 min, 0 % B. The flow was 1 ml/ml and all products were detected at 338 nm. Typical retention times were 4.2 min for the DTNB-cysteine adduct, 4.8 min for the DTNB-GSH adduct and 5.8 min for the free NTB. Quantification was performed using standards from incubations of DTNB (2 mM) with cysteine or GSH (each 10 or 100 µM).

Statistical Analysis
Results are expressed as mean±SEM. Two-way ANOVA (with Bonferroni’s correction for comparison of multiple means) was used for comparisons of vasodilator potency and efficacy. One-way ANOVA (with Bonferroni’s or Dunn’s correction for comparison of multiple means) was used for comparisons of constriction data, RONS detection, adhesion assays, as well as protein expression and modification studies. P values < 0.05 were considered significant.
Extended Discussion

In the present study we demonstrate that GPx-1 deficiency resulted in a phenotype of endothelial and vascular dysfunction that was substantially potentiated in aging. Importantly, both endothelium-dependent and -independent relaxation was impaired in aged GPx-1−/− mice. Altered eNOS function by inactivating or uncoupling phosphorylation and S-glutathionylation leading to diminished •NO-bioavailability are plausible explanations for this phenotype. The dysregulatory modifications of eNOS were also translated to increased uncoupling of the enzyme as envisaged by endothelial superoxide formation, which increased with age and was more pronounced in the GPx-1−/− group. In cultured endothelial cells we demonstrated that GPx-1 silencing increased adhesion of leukocytes, which may contribute to the observed endothelial/vascular dysfunction (e.g. by increased oxidative breakdown of •NO and/or impairment of the •NO-cGMP signaling cascade by infiltrated leukocytes). Furthermore, we observed an appreciable increase in cardiovascular oxidative stress and mild vascular remodeling, as detected by Sirius red and Masson's trichrome staining (indicative for increased fibrosis of the intima and thus a decrease in intima/media ratio).

When comparing the aged GPx-1 deficient mice with the aged wild type animals, striking differences in the spatial distribution of the 3-nitrotyrosine signal was noticed (see supplemental Figure S8). In aged GPx-1 deficient mice, a characteristic vascular distribution pattern of 3-nitrotyrosine within the intima, the media and in particular in the adventitia was evident, whereas the 3-nitrotyrosine was detected in vessels from aged wild type mice mainly in the intima. This could be related to direct effects of GPx-1 deficiency on infiltration of the vasculature with immune cells. Indeed, GPx-1 deficiency has been demonstrated to increase the susceptibility of cultured endothelial cells to lipopolysaccharide (LPS) by enforcing TLR4/CD14 signaling 21. In conductance vessels, sustained overproduction of vasodilators (e.g. NO by iNOS) may reduce the responsiveness of the vasculature to these messengers because of a desensitization of the NO/cGMP pathway 22. Indeed, increased iNOS expression and activity has been demonstrated for selenium-deficient RAW cell macrophages 23 and selenium is the precursor for selenocysteine synthesis forming the active site of GPx-1. iNOS-derived NO formation could also provide the basis for extensive protein tyrosine nitration as observed here in old mice in general and GPx-1 deficient mice in particular.

Link Between the Aging Process and Mitochondrial Oxidative Stress

Aging is an important cardiovascular risk factor for the development of atherosclerosis 24, 25, which can be accelerated by traditional risk factors such as diabetes, hypercholesterolemia, chronic smoking, hypertension, or obesity. A hallmark of vascular aging is the development of endothelial dysfunction 26 reflecting a decrease in vascular nitric oxide (•NO) bioavailability. The underlying mechanisms include increased production of reactive oxygen species (ROS) 27 with subsequent inactivation of •NO 28 and formation of reactive oxygen and nitrogen species (RONS), namely peroxynitrite 29. Elevated RONS may exhibit new messenger functions by post-translational oxidative modification of intracellular regulatory proteins 30 or may lead to irreversible alterations of biological macromolecules 31, 32. In this context, oxidative inactivation of prostacyclin synthase by peroxynitrite 33, which parallels the reported impaired endothelial prostacyclin formation with increasing age 34, may further contribute to age-related vascular dysfunction. Thus, aging-related induction and/or aggravation of endothelial dysfunction as well as increases in oxidative stress are of high clinical interest since both parameters represent predictors for the risk of future cardiovascular events 35, 36.

Previously, we provided evidence that mitochondrial oxidative stress increases with age and contributes to age-related endothelial/vascular dysfunction 37. Importantly, by using two genetic mouse models with ablated ALDH-2−/− or MnSOD−/− we could demonstrate that increased mitochondrial oxidative stress and oxidative mtDNA lesions are important
determinants for age-associated endothelial and vascular dysfunction. There is a large body of evidence for a link between cellular aging and mitochondrial dysfunction based on genetic animal models with increased mitochondrial ROS formation (e.g. MnSOD- or Trx-2-deficiency). Interestingly, overexpression of catalase, another enzyme crucially involved in the antioxidant defense, enhanced protection of mitochondria from RONS and extended life span in mice. The impact of antioxidant defense enzymes on aging-related cardiovascular complications has been previously demonstrated for SOD2, the cytosolic superoxide dismutase (SOD1), the extracellular superoxide dismutase (ecSOD), and thioredoxin-1 (Trx). However, no direct correlation between lifespan and most antioxidant enzymes (SOD2+/− or SOD2+/−xGPx-1−/−, GPx-1−/− or GPx-1−/−xMsrA−/−, Trx-2−/−, SOD1tg, catalasetg) could be observed since neither overexpression nor deletion of these genes affected the longevity. Only SOD1−/− mice and mice with double gene ablation combinations showed reduced life expectancy.

In the present study moderate differences between RONS formation in GPx-1−/− mice and their age-matched wild type littermates were observed. This rather moderate effect of GPx-1 deficiency on RONS levels in our model of vascular dysfunction is secondary to reduced expression of GPx-1 in the heart, muscle and aorta. Mice deficient in both MnSOD and GPx-1 (SOD2−/−xGPx-1−/−) showed no reduction in longevity despite increased oxidative damage and a greater incidence of pathology. This points towards effective compensatory mechanisms (e.g. upregulation of survival pathways) that allow these animals to survive despite the increased oxidative stress burden and resulting complications. Although GPx-1−/− mice reportedly do not compensate for their lack of GPx-1 by elevating catalase or SOD activities, they nonetheless show normal survival under laboratory conditions.

**Cellular Redox State, Aging Process, GPx-1 Deficiency and eNOS Function**

Cellular thiol levels and redox state are essential determinants of enzymatic function and accordingly of vascular function. However, intracellular GSH/GSSG levels are not easy to determine and previous literature demonstrated significant variances in the measurements of cellular GSH/GSSG levels in aged animals as well as rather marginal changes in thiol content in GPx-1−/− mice as compared to young or wild type controls. The substantial differences in reported GSH/GSSG ratio in young and aged animals are probably related to the type of investigated tissue but also to the employed detection method: 1) Ferrini et al. published GSH/GSSG ration of 180 in young aorta, 42 in old aorta, 975 in young tissue, 402 in old tissue, 16 in young blood and 5 in old blood. 2) Carvalho et al. reported on GSH/GSSG ration of 3.1 in young brain vessels, 1.9 in old brain vessels and 3.6 in young synaptosomes, 4.3 in old synaptosomes. This might be also largely attributed to the different expression levels of GSSG reductase and GPx-isoforms. According to previous reports, the GSH levels are not substantially different in GPx-1 containing (45 nmol/mg) or deficient (47 nmol/mg) astrocytes under resting conditions and same applies for GSH/GSSG ration in unstressed hepatocytes. However, upon oxidative challenges the levels of GSSG largely differ in GPx-1 containing cells due to utilization of GSH for detoxification of reactive species such as peroxides. Finally, Spector et al. observed no significant differences between non-protein thiol levels in the capsule epithelium of young (2.5 months) and old (24 months) animals, regardless of the presence or absence of the GPx-1 gene. In summary, the here presented reports make the cellular redox state (GSH/GSSG ratio) a very volatile parameter for the detection of mild oxidative stress.

Despite these drawbacks, we determined total cardiac tissue reduced thiol content by Elman’s reagent (5,5'-dithiobis-(2-nitrobenzoic acid), DTNB) dependent photometry (absorbance change at 430 nm) as well as HPLC-based quantification of DTNB-cysteine, DTNB-GSH adducts and free nitrothiobenzoate (NTB) upon size exclusion filtration (10 kDa) of the samples for non-protein thiols (NPT) (see supplementary Fig. S11). We observed a
moderate but consistent decline in reduced thiol groups in aged GPx-1-/- mice as compared to only a minor tendency of this decline in the aged B6 WT mice. Overall, the majority of literature supports a trend of decrease in reduced thiols during the aging process, which could affect the S-glutathionylation pattern and accordingly the coupling state of eNOS. Smith et al. showed in 2006 that the decline in endothelial GSH may contribute to a change of eNOS phosphorylation pattern (decline in P-Ser1176 and increase in P-Thr494) that was associated with a loss of vascular NO bioavailability, increased proinflammatory cytokines and impaired endothelium-dependent vasodilation. Recent work by Crabtree and coworkers even described an interplay of BH4 deficiency and eNOS S-glutathionylation in cells with diminished GTP-cyclohydrolase-1 expression providing a functional link between these two routes of eNOS uncoupling.
Extended References


Table S1. Collagen content determined from histochemical sirius red staining for vascular fibrosis in paraffinated aortic sections (6-7 spatially different measurements per cryo-section from 4-5 mice per group).

<table>
<thead>
<tr>
<th>Groups</th>
<th>B6 WT, 2mo</th>
<th>B6 WT, 6mo</th>
<th>B6 WT, 12mo</th>
<th>GPx-1-/-, 2mo</th>
<th>GPx-1-/-, 6mo</th>
<th>GPx-1-/-, 12mo</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Mean</strong></td>
<td>100,00</td>
<td>106,88</td>
<td>105,22</td>
<td>89,01</td>
<td>91,44</td>
<td>109,43</td>
</tr>
<tr>
<td><strong>SEM</strong></td>
<td>7,70</td>
<td>4,61</td>
<td>4,42</td>
<td>13,95</td>
<td>5,29</td>
<td>5,60</td>
</tr>
<tr>
<td><strong>n</strong></td>
<td>29</td>
<td>30</td>
<td>30</td>
<td>24</td>
<td>30</td>
<td>30</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>Statistical analysis</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>vs. B6 WT, 2mo</td>
</tr>
<tr>
<td>vs. B6 WT, 6mo</td>
</tr>
<tr>
<td>vs. B6 WT, 12mo</td>
</tr>
<tr>
<td>vs. GPx-1-/-, 2mo</td>
</tr>
<tr>
<td>vs. GPx-1-/-, 6mo</td>
</tr>
</tbody>
</table>

P = 0.443  P = 0.203  P = 0.474
P = 0.796  P = 0.032
P = 0.557
P = 0.861  P = 0.149
P = 0.023
Table S2. Aortic wall thickness determined from histochemical trichrom staining for vascular collagen fibrosis in paraffinized aortic sections (6-7 spatially different measurements per cryo-section from 4-5 mice per group).

<table>
<thead>
<tr>
<th>Groups</th>
<th>B6 WT, 2mo</th>
<th>B6 WT, 6mo</th>
<th>B6 WT, 12mo</th>
<th>GPx-1-/-, 2mo</th>
<th>GPx-1-/-, 6mo</th>
<th>GPx-1-/-, 12mo</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>75.02</td>
<td>90.03</td>
<td>92.20</td>
<td>67.41</td>
<td>84.34</td>
<td>99.34</td>
</tr>
<tr>
<td>SEM</td>
<td>3.63</td>
<td>2.54</td>
<td>2.44</td>
<td>1.43</td>
<td>2.17</td>
<td>2.82</td>
</tr>
<tr>
<td>n</td>
<td>30</td>
<td>30</td>
<td>29</td>
<td>30</td>
<td>30</td>
<td>26</td>
</tr>
</tbody>
</table>

Statistical analysis

- vs. B6 WT, 2mo: P = 0.001, P = <0.001, P = 0.056
- vs. B6 WT, 6mo: P = 0.542, P = 0.094
- vs. B6 WT, 12mo: P = 0.060
- vs. GPx-1-/-, 2mo: P = <0.001, P = <0.001
- vs. GPx-1-/-, 6mo: P = <0.001
**Figure S1.** Acetylcholine-dependent relaxation (endothelium-mediated) of isolated aortic ring segments and effect of preincubation with the BH₄ precursor sepiapterin (100 µM) and PEG-SOD (200 U/ml) for 1 h. Relaxation was assessed by isometric tension recording. The data are mean ± SEM of 5-6 aortic ring segments/group for sepiapterin and 16-19 for untreated samples (which were taken from Fig. 1A in the main manuscript). *, p<0.05 vs. respective young group (2mo).
Figure S2. GPx-1 deficiency aggravates vascular dysfunction during the aging process. (A) The sensitivity of aorta to the vasoconstrictor prostaglandin F2α (PGF2α) was assessed prior to measurements of the vasodilatory response. (B) Aortic protein expression of soluble guanylyl cyclase subunits and phosphorylation of VASP as a read-out for cGK-1 activity was detected by Western blot analysis. Representative blots are shown below the densitometric quantification. The data are mean ± SEM of 10-16 aortic ring segments from 6-9 animals/group (A) and 2 samples per group, each pooled from at least 3 different aorta (B). **, p<0.05 vs. indicated group. Statistical analysis was performed by t-test.
Figure S3. Aortic ratio of the Ser239 phosphorylated VASP and total VASP and the protein expression of the cGMP-dependent kinase cGK-1 was assessed by Western blotting using specific antibodies. The data are mean ± SEM of 3 aortic protein samples/group pooled from at least 6 animals/group. *, p<0.05 vs. B6 Ctr (2mo).
**Figure S4.** Iloprost-dependent relaxation (cAMP-mediated) of isolated aortic ring segments. Relaxation was assessed by isometric tension recording. The data are mean ± SEM of 4 animals/group. *, p<0.05 vs. B6 Ctr (2mo).
**Figure S5.** Histochemical sirius red staining for vascular fibrosis in paraffinized aortic sections. The staining colors collagen (red). From middle to left or right: lumen, endothelium, media, adventitia/adipose tissue. Magnification 20x. Representative for at least 3 animals.
Figure S6. Histochemical trichrom staining for vascular collagen fibrosis in paraffinated aortic sections. The staining colors collagen (turquoise blue). From middle to left or right: lumen, endothelium, media, adventitia/adipose tissue. Magnification 20x. Representative for at least 3 animals.
Figure S7. Cardiac NADPH oxidase activity was assessed by lucigenin (5 µM) ECL in isolated membranous fractions (0.2 mg/ml final protein) in the presence of NADPH (200 µM). Cardiac mitochondrial ROS formation was assessed by L-012 (100 µM) enhanced chemiluminescence (ECL) in isolated mitochondrial fractions (0.1 mg/ml final protein). The data are mean ± SEM of or 6-12 measurements with tissue from 3-6 animals/group (Nox activity) and 8-11 measurements with tissue pooled from at least 6 animals/group (mitochondria). Significance level as indicated or **, p<0.05 vs. indicated group.
Figure S8. Endothelial dihydroethidium (DHE, 1 µM) staining as a surrogate parameter for eNOS uncoupling. Representative microscope images are shown below the densitometric quantifications (more images are shown in the supplemental Figure S12). Some samples were preincubated with the BH₄ precursor sepiapterin (100 µM), the NOS inhibitor L-NAME (500 µM) or the Nox inhibitor VAS2870 (20 µM). Only the DHE fluorescence signal in the endothelial cell layer was quantified by densitometry. The data are mean ± SEM of 8-12 independent stainings for basal conditions and 4-8 independent stainings for the different treatments. Significance level as indicated or **, p<0.05 vs. indicated group.
Figure S9. Immunohistochemical staining for vascular Nox2 using a specific antibody in paraffinized aortic sections. Magnification 40x. E indicates the endothelial side of the vessel. Representative for at least 3 animals.
Figure S10. GPx-1 deficiency aggravates cardiovascular immunological staining for 3-nitrotyrosine- and 4-hydroxynonenal-positive proteins during the aging process. Cardiac oxidative stress was assessed by dot blot analysis for 3-nitrotyrosine- and 4-hydroxynonenal-positive proteins by using specific antibodies. Representative dot blots are shown at the bottom of each densitometric quantification graph. The data are mean ± SEM of 18-27 (3-NT) and 6 (4-HNE) protein samples from 6-9 (3-NT) and 3 (4-HNE) animals/group. *, p<0.05 vs. GPx-1−/− (2mo).
Figure S11. Aortic non-peptide carbonyl-groups in proteins was determined by “oxy” dot blot analysis upon derivatization with 2,4-dinitrophenylhydrazine and a specific antibody against 2,4-dinitrophenylhydrazone-protein adducts. The data are mean ± SEM of 5-6 animals/group. Significance level as indicated.
Figure S12. GPx-1 deficiency aggravates adverse eNOS regulation in the heart and aorta during the aging process. Phosphorylation of eNOS was determined by eNOS immunoprecipitation, followed by anti-Tyr657. Cardiac PYK-2 dependent phosphorylation of eNOS at Tyr657. Comparison of Tyr657 phosphorylation of eNOS in hearts from young and old wild type versus GPx-1−/− mice. Representative Western blots are shown below the densitometric quantification. After stripping the membrane the bands were stained for eNOS to allow normalization of the signals. The data are mean ± SEM of 4 (upper) and 2 (lower) protein expression assays from 4-6 animals/group. *, p<0.05 vs. GPx-1−/− (2mo) and #, p<0.05 vs. GPx-1−/− (6mo).
Figure S13. GPx-1 deficiency aggravates adhesion of human leukocytes to cultured human endothelial cells and induces a pro-inflammatory phenotype during the aging process. EA.hy 926 cells (Ctr, siCtr and siGPx-1) were incubated with human PMN (1x or 5x10⁵ cells/ml) (A) or human monocytes/lymphocytes (1x or 5x10⁵ cells/ml) (B) and LPS (5 or 25 µg/ml) termed as low and high conditions. Adherent PMN were quantified by phorbol ester induced oxidative burst measured by L-012 ECL. The RONS signal was increased in GPx-1 silenced endothelial cells (A). This signal further increased with the number of incubated polymorphonuclear neutrophils (PMN) and their stimulation by LPS. The GPx-1 silenced groups always showed the highest RONS signal, indicating the highest number of adherent PMN. Comparable results were obtained with isolated human monocytes/lymphocytes (WBC) and cultured endothelial cells (B). The data are mean ± SEM of at least 5 (A-B) independent experiments/group. Panel A-B: all tested groups were significantly different against the other groups.
Figure S14. Adventitial ROS formation was determined by adventitia-specific densitometric quantification of the aortic dihydroethidium-derived fluorescence signal. The data are mean ± SEM of 6-7 animals/group. Significance level as indicated or **, p<0.05 vs. indicated group.
Figure S15. Determination of cellular levels of reduced thiol-groups by Ellman’s reagent. (A) hearts were homogenized in 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB, 1 mM), diluted 1:40 in DTNB (0.5 mM) and after 5 min the difference in absorbance at 430 nm was measured. (B) Representative chromatograms of HPLC analysis of DTNB-cysteine adduct, DTNB-GSH adduct and free nitrothiobenzoate (NTB). All products were detected at 338 nm. (C) Hearts were homogenized, centrifuged, the supernatant filtrated and the low molecular weight thiols were incubated with DTNB (2 mM) for 5 min and the samples were subjected to HPLC analysis. Below the quantification of each measured product the respective representative magnified chromatograms are shown. All values were normalized to the tissue wet weight. The data are mean ± SEM of pooled hearts from 2-3 (A) and 5-7 animals/group.
<table>
<thead>
<tr>
<th>B6_1mth</th>
<th>B6_6mth</th>
<th>B6_12mth</th>
<th>GPx⁻/⁻_1mth</th>
<th>GPx⁻/⁻_6mth</th>
<th>GPx⁻/⁻_12mth</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image1.png" alt="Image" /></td>
<td><img src="image2.png" alt="Image" /></td>
<td><img src="image3.png" alt="Image" /></td>
<td><img src="image4.png" alt="Image" /></td>
<td><img src="image5.png" alt="Image" /></td>
<td><img src="image6.png" alt="Image" /></td>
</tr>
<tr>
<td><img src="image7.png" alt="Image" /></td>
<td><img src="image8.png" alt="Image" /></td>
<td><img src="image9.png" alt="Image" /></td>
<td><img src="image10.png" alt="Image" /></td>
<td><img src="image11.png" alt="Image" /></td>
<td><img src="image12.png" alt="Image" /></td>
</tr>
<tr>
<td><img src="image13.png" alt="Image" /></td>
<td><img src="image14.png" alt="Image" /></td>
<td><img src="image15.png" alt="Image" /></td>
<td><img src="image16.png" alt="Image" /></td>
<td><img src="image17.png" alt="Image" /></td>
<td><img src="image18.png" alt="Image" /></td>
</tr>
<tr>
<td><img src="image19.png" alt="Image" /></td>
<td><img src="image20.png" alt="Image" /></td>
<td><img src="image21.png" alt="Image" /></td>
<td><img src="image22.png" alt="Image" /></td>
<td><img src="image23.png" alt="Image" /></td>
<td><img src="image24.png" alt="Image" /></td>
</tr>
<tr>
<td><img src="image25.png" alt="Image" /></td>
<td><img src="image26.png" alt="Image" /></td>
<td><img src="image27.png" alt="Image" /></td>
<td><img src="image28.png" alt="Image" /></td>
<td><img src="image29.png" alt="Image" /></td>
<td><img src="image30.png" alt="Image" /></td>
</tr>
<tr>
<td><img src="image31.png" alt="Image" /></td>
<td><img src="image32.png" alt="Image" /></td>
<td><img src="image33.png" alt="Image" /></td>
<td><img src="image34.png" alt="Image" /></td>
<td><img src="image35.png" alt="Image" /></td>
<td><img src="image36.png" alt="Image" /></td>
</tr>
<tr>
<td><img src="image37.png" alt="Image" /></td>
<td><img src="image38.png" alt="Image" /></td>
<td><img src="image39.png" alt="Image" /></td>
<td><img src="image40.png" alt="Image" /></td>
<td><img src="image41.png" alt="Image" /></td>
<td><img src="image42.png" alt="Image" /></td>
</tr>
<tr>
<td><img src="image43.png" alt="Image" /></td>
<td><img src="image44.png" alt="Image" /></td>
<td><img src="image45.png" alt="Image" /></td>
<td><img src="image46.png" alt="Image" /></td>
<td><img src="image47.png" alt="Image" /></td>
<td><img src="image48.png" alt="Image" /></td>
</tr>
</tbody>
</table>

50 μm
**Figure S16.** Oxidative dihydroethidine fluorescence microtopography in aortic cryo sections. Additional representative fluorescence micrographs (black and white) for the whole aortic wall DHE fluorescence (upper panel) and for the endothelial cell layer only (lower panel). The scale bar in the upper left image of whole aortic wall DHE staining represents 50 µM and also accounts for the endothelial DHE stainings. The magnification was 400x.