Combined Deficiency in Glutathione Peroxidase 4 (Gpx4) and Vitamin E Causes Multi-Organ Thrombus Formation and Early Death in Mice

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ABSTRACT

Rationale: Growing evidence indicates that oxidative stress contributes markedly to endothelial dysfunction. The selenoenzyme glutathione peroxidase 4 (Gpx4) is an intracellular antioxidant enzyme important for the protection of membranes by its unique activity to reduce complex hydroperoxides in membrane bilayers and lipoprotein particles. Yet a role of Gpx4 in endothelial cell function has remained enigmatic.

Objective: To investigate the role of Gpx4 ablation and subsequent lipid peroxidation in the vascular compartment in vivo.

Methods and Results: Endothelial-specific deletion of Gpx4 had no obvious impact on normal vascular homeostasis nor did it impair tumor-derived angiogenesis in mice maintained on a normal diet. By stark contrast, aortic explants from endothelial-specific Gpx4 knockout mice showed a markedly reduced number of endothelial branches in sprouting assays. To shed light onto this apparent discrepancy between the in vivo and ex vivo results, we depleted mice of a second antioxidant, vitamin E, which is normally absent under ex vivo conditions. Mice were therefore fed a vitamin E-depleted diet for 6 weeks before endothelial deletion of Gpx4 was induced by 4-hydroxytamoxifen. Surprisingly, about 80% of the knockout mice died. Histopathological analysis revealed detachment of endothelial cells from the basement membrane as well as endothelial cell death in multiple organs which triggered thrombus formation. Thromboembolic events were the likely cause of various clinical pathologies including heart failure, renal and splenic micro-infarctions or paraplegia.

Conclusions: Here we show for the first time that in the absence of Gpx4, sufficient vitamin E supplementation is crucial for endothelial viability.

Keywords: Oxidant stress, thrombosis, vascular endothelium, vascular endothelial function, α-Tocopherol

Nonstandard Abbreviations and Acronyms:

- AIF: apoptosis inducing factor
- ApoE−/−: apolipoprotein E-deficient mice
- EC: endothelial cell
- eEPC: embryonic endothelial progenitor cell
- Gpx4: glutathione peroxidase 4
- Gpx4ECKO: tamoxifen-inducible endothelial-specific Gpx4 knockout mice
- Gpx4control: control littermates to Gpx4ECKO mice
- H2O2: hydrogen peroxide
- LLC1: Lewis Lung Carcinoma cells
- MDA: malondialdehyde
- MEF: mouse embryonic fibroblasts
- PHGPx: phospholipid hydroperoxide glutathione peroxidase
- SMC: smooth muscle cells
- TAM: Tamoxifen
- TEM: transmission electron microscopy
- VTE: venous thromboembolism
INTRODUCTION

Peroxidation of lipids and the formation of bioactive lipid peroxidation products have been implicated in a number of pathophysiological processes, including inflammation and atherogenesis.\(^1\,2\) The selenoenzyme glutathione peroxidase 4 (Gpx4), one of eight glutathione peroxidases in mammals, is an intracellular antioxidant enzyme unique for its activity to reduce phospholipid hydroperoxides in membrane bilayers.\(^3\,4\) In addition, Gpx4 can react with hydrogen peroxide (H\(_2\)O\(_2\)) and a wide range of lipid hydroperoxides, including those derived from lipoprotein particles as well as from cholesterol and cholesteryl esters.\(^4\,6\) Disruption of Gpx4 in the mouse leads to early embryonic lethality at embryonic day 7.5.\(^7\,8\) Inducible mouse embryonic fibroblasts (MEFs) isolated from conditional Gpx4 knockout mice die shortly after knockout induction.\(^9\) Cell death progression downstream of Gpx4 inactivation was linked to increased 12/15 lipoxygenase-derived lipid peroxidation rather than accumulation of water-soluble oxygen radicals. Increased lipid peroxidation causes cell death in a caspase-independent, apoptosis-inducing factor (AIF)-mediated manner.\(^9\) Remarkably, cell death of inducible Gpx4 knockout MEFs and primary neurons could be prevented by supplementation of the lipophilic antioxidant vitamin E (α-tocopherol). By contrast, water-soluble antioxidants proved to be largely ineffective, indicating that Gpx4 acts mainly at the membranous compartment. In accordance, transformed Gpx4 deleted MEFs died in vitro following knockout induction.\(^10\) However, when implanted subcutaneously into wild-type mice these cells survived and formed tumors. This suggests that a less toxic, more protective environment is present in vivo to which higher levels of lipophilic antioxidants in serum may contribute.\(^10\)

Since the ability of cells to up-regulate intrinsic anti-oxidant enzymes is fundamentally important in protecting cells from enhanced oxidative stress,\(^11\,13\) several studies addressed whether over-expression of Gpx4 might also increase the cell’s resistance against high levels of lipid hydroperoxides. Indeed, Gpx4 over-expression in smooth muscle cells (SMCs) lowered oxLDL-induced proliferation compared to un-transfected SMCs.\(^14\) In accordance, over-expression of Gpx4 in apolipoprotein E-deficient mice (ApoE\(^-\)) inhibited the development of atherosclerosis by decreasing lipid peroxidation.\(^15\) Remarkably, such mice displayed a diminished number of dying endothelial cells (ECs). Furthermore, high amounts of Gpx4 markedly decreased the sensitivity against H\(_2\)O\(_2\)-induced cytotoxicity in ECs in vitro.\(^16\) These results indicate the importance of Gpx4 in maintaining endothelial cell function and vascular wall integrity and prompted us to study the yet unexplored role of this redox enzyme in resting as well as proliferating ECs in vivo.

In view of the fact that oxidative stress and augmented lipid peroxidation have been identified as important detrimental determinants in cardiovascular diseases, we were surprised that endothelial loss of Gpx4 produced no obvious phenotype, neither under baseline conditions nor under forced angiogenesis following tumor cell implantation. However, the combined loss of endothelial Gpx4 expression plus dietary depletion of the lipophilic antioxidant vitamin E dramatically impaired vascular homeostasis and resulted in multifocal thrombus formation. Our study reveals that in the absence of Gpx4, vitamin E is essentially required for the homeostasis of blood vessels in the adult mouse.

METHODS

Mice.

To analyze the biological significance of Gpx4 expression in vascular endothelial cells in vivo, we interbred Gpx4\(^{lox/lox}\) mice\(^9\) with transgenic mice expressing the tamoxifen-inducible recombinase CreERT2 under control of the endothelial Cdh5 promoter (a kind gift from Dr. Ralf Adams, MPI Münster).\(^17\) These mice were further crossed with Gpx4\(^{lox/lox}\), Gpx4\(^{lox/wt}\) and Gpx4\(^{wt/wt}\) mice to generate litters containing Gpx4\(^{lox/lox}\)/Cdh5(PAC)-CreERT2 (referred to as Gpx4\(^{iECKO}\)) and control litters (referred to Gpx4\(^{control}\)) (Figure 1A). Control litters include mice of the following genotypes: Gpx4\(^{lox/lox}\), Gpx4\(^{lox/wt}\), and Gpx4\(^{wt/wt}\) Cdh5(PAC)-CreERT2. To activate Cre recombinase in mice carrying the Cdh5(PAC)-CreERT2 transgene, 4-hydroxy-tamoxifen (T5648, Sigma-Aldrich,}

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Deisenhofen, Germany) was administered by oral gavage on 5 consecutive days followed by a final sixth oral application one week thereafter (30 µg tamoxifen per g body weight per day, diluted in corn oil (C8267, Sigma-Aldrich). The same tamoxifen treatment protocol was applied to control mice.

All mice were kept under standard conditions with food (ssniff, Soest, Germany) and water ad libitum. Mice with dietary depletion of vitamin E received a special diet (# 15791-147 vitamin E-depleted diet (ssniff) containing 7 mg/kg vitamin E as compared to 55 mg/kg in normal chow) for at least six weeks before knockout induction. In a second set of experiments the order of the events was inverted: First, endothelial specific Gpx4 deletion was induced. Thereafter, the dietary depletion of vitamin E was initiated 6 weeks later. In both settings the vitamin E-depleted diet was applied to knockout as well as control mice. Animal experiments were performed in compliance with the German Animal Welfare Law and had been approved by the institutional committee on animal experimentation and the government of Upper Bavaria.

**Isolation of endothelial cells from heart and lung tissue.**

Two weeks following the last tamoxifen application, ECs isolated from heart and lung tissue were analyzed by western blotting to confirm the loss of endothelial Gpx4 expression.

Isolation of ECs was performed using a rat anti-mouse CD31 antibody (1:50, BM 4086, Acris Antibodies GmbH, Herford, Germany), goat anti-rat IgG Micro Beads (20 µl per 10^7 cell, #130-048-501, Miltenyi Biotec, Bergisch Gladbach, Germany) and MACS MS Columns (#130-042-201, Miltenyi Biotec) according to the manufacturer's manual.

**Depletion of endothelial cells from brain tissue.**

One hemisphere of the brain (3 Gpx4^kieKO and 3 Gpx4^control mice) was cut into pieces of approximately 1 mm^3 and homogenized using the Neural Tissue Dissociation Kit (130-092-628, Miltenyi Biotec). The single cell solution was filtered through a 70 µm cell strainer, rinsed three times and centrifuged at 400 x g for 10 minutes at 4°C. Depletion of the ECs was accomplished using a rat anti-mouse CD31 antibody (1:50, BM4086, Acris Antibodies), goat anti-rat IgG Micro Beads (20 µl per 10^7 cells, Miltenyi Biotec) and MACS LD columns (#130042901, Miltenyi Biotec) according to manufacturer’s recommendations.

**Immunoblotting.**

Detection of Gpx4 in isolated ECs was achieved with a monoclonal peptide antibody specific for Gpx4. The monoclonal rat C-terminal antibody 1B4 was produced as described previously. Each blot was reprobed for actin (1:1000, A2066, Sigma-Aldrich). Semiquantitative analysis was performed using the Wasabi imaging software (Hamamatsu Photonics Deutschland, Herrsching, Germany).

**Cell lines and reagents.**

Lewis Lung Carcinoma cells (LLC1) were cultured at 37°C under 5% CO2 and 5% O2 in Dulbecco’s modified Eagle medium (#41965-039, Invitrogen, Karlsruhe, Germany) supplemented with 10% fetal bovine serum (S 0115 Biochrom, Berlin, Germany), 2 mM L-glutamine (#25030032, Invitrogen) and 100 U/ml penicillin/streptomycin (#15140-122, Invitrogen).

**Histology.**

Five-micrometer sections of 4% (w/v, in PBS) paraformaldehyde-fixed and paraffin-embedded material were stained with hematoxylin-eosin (H&E) as described or with the Masson-Goldner staining kit (#100485, Merck Millipore, Billerica, MA, USA) according to the manufacturer’s instructions.

**Immunohistochemistry and image analysis.**

Organ samples as well as tumor tissue samples were snap frozen in liquid nitrogen and stored at -80°C. Immunohistochemistry and immunofluorescence were performed as described previously. Immunoassaying was analyzed using the Olympus BX41 microscope in combination with the digital camera CAMEDIA C-5050 and the software Olympus DP-Soft v3.2 (Olympus, Tokio, Japan).
**Ex vivo mouse aortic ring angiogenesis assay.**

*Ex vivo* angiogenesis was studied by culturing mouse aortic rings in a three-dimensional Matrigel matrix. Aortic rings of **Gpx4iECKO** mice as well as control littermates were cultured in the presence or absence of (±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox; 1 μM, #56510; Sigma-Aldrich), a water-soluble analog of vitamin E.10, 20

**Embryonic endothelial progenitor cells.**

In order to gain further insight into the role of Gpx4 in the endothelial compartment, we employed embryonic endothelial progenitor cells (eEPC) which were isolated from **Gpx4lox/lox** and **Gpx4wt/lox** mouse embryos at embryonic day 7.5.21 These cells were stably transfected with tamoxifen-inducible MERCreMER (MER, mutated estrogen receptor) as described.9

To maintain the viability of cells after inducing the deletion of Gpx4 by tamoxifen treatment, cells were cultured in the presence of 1 μM Trolox (Sigma-Aldrich).

**Quantification of blood vessel density.**

Blood vessel density, defined as percentage of CD31-positive staining per area, was quantified using pixel-based thresholds in a computer-assisted image analysis software (KS400 Image System; Carl Zeiss Vision, Jena, Germany).

**Quantification of ex vivo angiogenesis.**

Microvessel outgrowth was studied with an Olympus microscope at appropriate magnification using phase-contrast microscopy. Image analysis was performed with the software Aqual as described before.22 After generation of a binary image, the following semi-automatic measurements were performed: the number of microvessels; the maximal microvessel length, and the total number of branches.

**Hemodynamic parameters and endothelial function in vivo.**

Arteriolar resting tone and endothelial function were investigated in arterioles of the cremaster muscle and calculated as described previously.23

**Determination of serum malondialdehyde (MDA) levels by high-performance liquid chromatography (HLPC).**

Blood samples were obtained from the facial vein 10 days after the final tamoxifen application. Whole blood samples were kept at room temperature for 20 min followed by centrifugation at 3000 x g for 10 min at 4°C. The serum was collected and HLPC analysis performed as described previously.24, 25

**Statistical analysis.**

Statistical analysis was performed using SigmaStat© 2.0 software (Jandel GmbH, Erkrath, Germany). Experimental values are expressed as mean ± SEM, unless otherwise stated. Statistically significant differences between groups were calculated by Student's *t*-test or ANOVA followed by Bonferroni's correction. Non-Gaussian distributed data were analyzed by the non-parametric Kruskal-Wallis test for non-paired data. *P* < 0.05 was considered significant.

**RESULTS**

*Generation and histopathological analysis of Gpx4<sup>iECKO</sup> mice.**

The breeding strategy for the generation and genotyping of **Gpx4<sup>iECKO</sup>** mice is depicted in Figure 1A,B. Two weeks following the last tamoxifen application, endothelial loss of Gpx4 expression was verified in endothelial cells isolated from either heart or lung tissue by western blotting (Figure 1C). Semi-quantitative analysis of endothelial Gpx4 expression revealed a 75% reduction in **Gpx4<sup>iECKO</sup>** (100% in control mice vs. 24.8 +/- 4.0% in **Gpx4<sup>iECKO</sup>** mice; endothelial Gpx4 expression of control mice was arbitrarily set to 100%). In contrast, following the depletion of endothelial cells from brain...
tissue, no difference in Gpx4 expression was detectable between Gpx4ECKO and control mice by western blot analysis, indicating that non-endothelial tissue is not affected by the endothelium-specific deletion of Gpx4 (100% in Gpx4control and 108 +/- 15% in Gpx4ECKO mice, Gpx4 expression of control mice was arbitrarily set to 100%). Gpx4ECKO mice developed no obvious phenotype within six months (the maximum observation period) following knockout induction. Survival rate was analyzed for 15 Gpx4ECKO mice as well as 21 control mice. The control group involved the following genotypes: Gpx4lox/lox (10 animals), Gpx4lox/wt (2 animals) and Gpx4lox/wt Cdh5(PAC)-CreERT2 (9 animals). Immunohistological analysis of the vasculature of various organs (e.g. heart, liver, lung, spleen, brain, kidney) did not reveal any morphological abnormalities in response to endothelial Gpx4 deletion such as vessel density and/or vessel integrity (exemplarily shown for heart and renal tissue in Figure 1D,E).

Tumor growth and angiogenesis is not hindered in Gpx4ECKO mice.

Since endothelial deletion of Gpx4 did not result in any obvious impairment of vascular homeostasis, we addressed whether it might affect the growth of new blood vessels using a tumor model to study tumor-derived angiogenesis. Subcutaneous implantation of LLC1 tumor cells into Gpx4ECKO, as well as control littermates, resulted in tumors of similar volume and mass (Figure 2A). Quantification of vascularization by CD31 immunohistochemistry revealed no differences between the experimental groups, neither in vascular density (Figure 2B) nor in the number of vascular structures (data not shown).

Aortic explants derived from Gpx4ECKO mice show significantly impaired branching.

Although the adult vasculature in induced Gpx4ECKO mice revealed no abnormal morphology, we asked whether a difference might be unmasked ex vivo. Therefore, aortic explants from Gpx4ECKO (n=5) and control littermates (n=5) were cultured for a period of 10 days (Figure 3A). While the decrease in the number of sprouts from aortic explants of Gpx4ECKO mice as compared to controls was not statistically significant (Figure 3B), a significant reduction in both the number of branches (Figure 3C) as well as sprout length was detectable in the knockout explants (Figure 3D).

In a second set of experiments, we tested whether the addition of Trolox, a water-soluble analog of the lipophilic antioxidant vitamin E, to the cell culture medium was able to restore a normal branching pattern in aortic rings derived from Gpx4ECKO mice (Online Figure I). Trolox supplementation rescued the number of branches in knockout explants compared to non-treated explants to a significant extent (Online Figure IA, B). The increase in the overall sprout length observed upon Trolox treatment did, however, not reach statistical significance (Online Figure ID).

Inducible depletion of Gpx4 in mouse eEPCs leads to massive cell death.

We have shown previously that disruption of the Gpx4 gene in mouse embryonic fibroblasts (MEFs) and in c-myc/ha-ras transformed MEFs caused rapid cell death. As a more relevant cellular model for endothelial cells we generated a tamoxifen inducible knockout cell system using eEPCs harboring one or two loxP flanked Gpx4 alleles (Online Figure IIA, B). These cells were subsequently transfected with MerCreMer, allowing the tamoxifen-dependent inducible depletion of Gpx4. TAMoxifen treatment induced cell death in Gpx4lox/lox but not in Gpx4wt/lox eEPCs within 72 hours (Online Figure IIC), which could be fully compensated by Trolox ruling out deleterious side-effects of tamoxifen (Online Figure IID).

Vitamin E deprivation in vivo causes sudden death or paralysis in a fraction of Gpx4ECKO mice.

Considering that Gpx4-mediated cell death in eEPCs could be prevented by vitamin E and that ex vivo cultured ECs from Gpx4ECKO mice revealed marked defects in vascular branching, whereas endothelial-specific Gpx4 knockout mice show no obvious vascular defects, we asked whether the vitamin E content in the chow may compensate for endothelial Gpx4 deletion. In order to address this we replaced the normal mouse diet, which is generally enriched in vitamin E (55 mg/kg vitamin E), by a vitamin E-deprived diet (7mg/kg vitamin E) for at least 6 weeks before deletion of endothelial Gpx4.
Strikingly, 80% of the Gpx4\textsuperscript{ECKO} mice maintained on this vitamin E-deprived diet died within three weeks or suffered from severe paralysis (three out of 18) following the final tamoxifen application (Figure 4) and had to be sacrificed.

In the protocol used above deletion of Gpx4 is induced by tamoxifen-induced activation of Cre recombinase under conditions of dietary vitamin E deprivation. Ubiquitous transient Cre activation has been shown to induce double strand breaks and growth inhibition in mammalian cells in vitro\textsuperscript{26} and severe toxicity in mice in vivo\textsuperscript{27}, which may dramatically aggravate the phenotype when the gene of interest is deleted. To rule out the possibility that Cre toxicity may be responsible for the phenotype or may severely contribute to a composite phenotype in combination with Gpx4 loss and vitamin E deprivation, we inverted the order of events. After endothelial-specific deletion of Gpx4, mice were allowed to recover from Cre toxicity and to adapt to the deletion of Gpx4 for 6 weeks before vitamin E was restricted. The inverted protocol resulted in a slightly alleviated phenotype. Only one sudden death was observed in the group of Gpx4\textsuperscript{ECKO} mice (10 animals). Five other mice had to be sacrificed since their general condition was poor. One of these mice suffered from myocardial infarction, one from hemiparesis, another showed forelimb paralysis. The two remaining mice demonstrated multiple micro-thrombotic events in the kidneys. In the control group one animal (Gpx4\textsuperscript{lox/lox}) suffered from bite injury and had to be euthanized, all other mice survived without developing any symptoms (observation period: 6 months). The control group was composed of the following animals: Gpx4\textsuperscript{lox/lox} (10 animals), Gpx4\textsuperscript{lox/wt} (1 animal) and Gpx4\textsuperscript{lox/wt} Cdh5(PAC)-CreERT2 (5 animals) (Online Figure III). Although we cannot formally discriminate between an adaptive response (activation of other enzymatic antioxidant systems other than Gpx4 after genetic ablation of Gpx4 or aggravation of the phenotype by the combined toxic action of Cre recombinase activity in concert with Gpx4 deletion and vitamin E depletion), this experiment allowed to make two crucially important points: firstly, there is no other antioxidant system than vitamin E that might take over the function of Gpx4 after adaptation to Gpx4 deletion, and secondly, Cre toxicity could be definitively ruled out as critical culprit of the phenotype.

\textit{Vitamin E-low diet increases the risk of thrombosis in Gpx4\textsuperscript{ECKO} mice.}

Due to paraplegia of some mice (two out of 18 mice suffered from hindlimb paralysis, 1 out of 18 from forelimb paralysis), we hypothesized that thrombus formation might be one of the underlying reasons for paraplegia as well as sudden death in Gpx4\textsuperscript{ECKO} mice. Indeed, a systemic histological analysis of different organs confirmed our assumption (Figure 5). One possible reason for the thromboembolic events in Gpx4\textsuperscript{ECKO} mice might be endothelial cell death because dying ECs are known to be procoagulant.\textsuperscript{28} Therefore, we analyzed sections from multiple organs by combined CD31/TUNEL staining. In fact, single TUNEL-positive endothelial cells were detectable in lung, kidney, liver and heart tissue of Gpx4\textsuperscript{ECKO} mice (exemplarily shown for heart and renal tissue in Figure 5; left column, top and bottom line), which was in sharp contrast to control littermates where no TUNEL-positive endothelial cells could be detected.

Immunofluorescence staining with an antibody against CD41 further verified platelet aggregation and thrombus formation in various organs (exemplarily shown for heart and renal tissue in Figure 5; middle columns, top and bottom line). Such thrombus formation resulted in multiple pathologies like myocardial infarction (Figure 5, right column, top line) or micro-infarctions in the kidney (Figure 5, right column, bottom line). Micro-infarctions as well as micro-bleedings could also be detected in various organs and tissues such as spleen, liver and others (not shown). Intriguingly, paralyzed mice had micro-bleedings within the spinal cord (Figure 6A). Further analysis of the spinal cord and the spinal nerves revealed multiple intravascular thrombi (insert in Figure 6B). Such thrombus formation might be responsible for the observed spinal nerve degeneration (Figure 6B), which was not detectable in Gpx4\textsuperscript{control} mice (Figure 6C).

\textit{Mean arterial blood pressure (MAP) and heart rate.}

To address whether the endothelium-specific disruption of Gpx4 in combination with vitamin E depletion has any functional consequences on the vascular tone, MAP and heart rates were
determined. MAP was significantly elevated in Gpx4<sup>IECKO</sup> mice compared to control mice (79 ± 2 mmHg vs. 69 ± 3 mmHg respectively, n=6 each, p<0.05). In contrast, heart rate was not different between Gpx4<sup>IECKO</sup> mice and control mice (226 ± 13 bpm vs. 218 ± 35 bpm, respectively) (Table 1).

**Resting arteriolar tone and acetylcholine induced dilation.**

A total of 76 arterioles from 7 control mice and 79 arterioles from 6 Gpx4<sup>IECKO</sup> mice were investigated. Maximal diameters of the arterioles were not significantly different between control littermates (38 µm, range 18-69 µm) and Gpx4<sup>IECKO</sup> mice (35 µm, range 16-70 µm). The arterioles in untreated preparations showed varying normalized diameters (from 0.25 to 1.00); however, normalized diameter was lower in Gpx4<sup>IECKO</sup> mice compared to control littermates (0.78 ± 0.02 vs. 0.89 ± 0.02, p<0.05), corresponding to an increased arteriolar resting tone. In contrast, acetylcholine dependent vasodilation (expressed as % of maximal vessel dilation) as an indicator of endothelial function was not significantly different in Gpx4<sup>IECKO</sup> mice compared to control mice, neither in response to low acetylcholine (1 µmol/L) (24 ± 5% vs. 17 ± 4% in control mice), nor in response to high acetylcholine concentrations (10 µmol/L) (83 ± 3% vs. 75 ± 3% in control mice). Correspondingly, acetylcholine dependent vasodilation (expressed as % of maximal vessel dilation) as an indicator of endothelial function was not significantly different in Gpx4<sup>IECKO</sup> mice compared to control mice, neither in low acetylcholine (1 µmol/L) (24 ± 5% vs. 17 ± 4% in control mice), nor in response to high acetylcholine concentrations (10 µmol/L) (83 ± 3% vs. 75 ± 3% in control mice). Correspondingly, the response to 1 µmol/L of norepinephrine (which was used to normalize the vessel tone for the investigation of acetylcholine-induced vasodilation) was similar in Gpx4<sup>IECKO</sup> and control littermates (normalized vessel diameter after norepinephrine 0.48 ± 0.02 in Gpx4<sup>IECKO</sup> mice vs. 0.47 ± 0.02 in controls), respectively. Results are shown in Table 1.

**Increased lipid peroxidation in vitamin E-depleted Gpx4<sup>IECKO</sup> mice.**

Malondialdehyde (MDA) is one of the most established indicators of lipid peroxidation. MDA determinations by HPLC of freshly collected blood samples from mice kept on a standard diet revealed no difference in the MDA levels between control mice (n=6) and Gpx4<sup>IECKO</sup> littermates (n=6) (13.06 ± 2.25 µM vs. 11.71± 2.14 µM). However, when mice received a vitamin E-depleted diet, in Gpx4<sup>IECKO</sup> mice significantly higher MDA plasma levels were observed (14.7± 3.07 µM in control mice vs. 20.04± 2.87 µM in Gpx4<sup>IECKO</sup> mice) (Figure 6D).

**Vitamin E depletion leads to endothelial cell ablation in Gpx4<sup>IECKO</sup> mice.**

Analysis of the aorta as well as of renal glomeruli by transmission electron microscopy revealed a partial ablation of ECs from the basement membrane in Gpx4<sup>IECKO</sup> mice kept under a vitamin E-depleted diet (Figure 7, right column). Control littermates which were also kept on a vitamin E-depleted diet revealed no obvious phenotype (Figure 7, left column) except that single mitochondria in renal endothelial cells appeared swollen.

**DISCUSSION**

The endothelium maintains vascular homeostasis through multiple complex interactions with cells in the vessel wall and vessel lumen. Considerable evidence suggests that oxidative stress is an important contributing factor in endothelial dysfunction. The functional role of endothelial-expressed Gpx4 in the maintenance of vascular homeostasis in vivo has not been explored to date.

Hence, we strived to uncover the biological significance of Gpx4 as well as its interplay with vitamin E in resting as well as proliferating ECs in vivo. Interestingly, vascular integrity was not affected when Gpx4 alone was deleted in the endothelium. Similarly, tumor-induced angiogenesis in Gpx4<sup>IECKO</sup> mice was comparable to wild-type littermates, indicating an efficient back-up system under in vivo conditions. Two observations led us to the assumption that the back-up system might be available only in vivo, but not in ex vivo cell systems: firstly, Gpx4<sup>IECKO</sup> mice display significantly impaired angiogenesis in an ex vivo aortic ring angiogenesis assay, and secondly, Gpx4 knockout eEPCs rapidly die when explanted in culture. Indeed, while vitamin E is not a component of the Endothelial Cell Growth Medium, the applied mouse diet is highly supplemented with vitamin E. In order to substantiate our hypothesis that vitamin E in the diet is masking the genetic loss of Gpx4 in the mouse diet...
vascular ECs in vivo, mice were fed a vitamin E-depleted diet for at least six weeks before deletion of Gpx4. Strikingly, the combined reduction of vitamin E and loss of endothelial Gpx4 expression resulted in fatal outcome in the majority of mice. Approximately 80% of the Gpx4iECKO mice suffered either from paralysis (three out of 18) or suddenly died within three weeks after the final tamoxifen application (14 out of 18). To allow adaptation of the mice to Gpx4 deletion in the endothelial system and to exclude toxic side effects of induced Cre recombinase activity, the order of events was inverted. After deletion of Gpx4 by tamoxifen administration, mice were allowed to adapt and to recover from Cre toxicity for six weeks, before the vitamin E-depleted diet was initiated. Using this reverse protocol a mitigated phenotype was observed (delayed onset and fewer affected animals). Nonetheless, the same pathological findings could be demonstrated in more than half of the animals (6 out of 10). The confirmation of the phenotype in the reciprocal protocol provided definitive proof for the backup function of vitamin E for Gpx4 deficiency in endothelial cells in vivo and excluded Cre toxicity as a putative culprit in provoking the observed phenotype. Nevertheless, because there was a severity difference between the two groups, we cannot absolutely exclude that Cre activation exacerbated the phenotype. Since the induced deletion of Gpx4 results in lipid peroxidation-induced cell death in different kinds of cultured cells,9, 10, 32 we hypothesized that Gpx4iECKO mice may also suffer from increased lipid peroxidation. As expected, serum levels of malondialdehyde, the most frequently used biomarker for enhanced lipid peroxidation,29 were elevated in the serum of vitamin E-depleted Gpx4iECKO mice, but not in serum of control littermates or of Gpx4iECKO mice kept on a standard diet. Because several lipid peroxidation derivatives are known to induce EC apoptosis,33, 34 we analyzed whether we could detect dying ECs. Indeed, TUNEL-positive ECs were observed in different organs of vitamin E-depleted Gpx4iECKO mice but not in organs of control mice or of mice on a standard diet. Detailed analysis of EC morphology by TEM supported this observation. Vitamin E-depleted Gpx4iECKO mice revealed a partial detachment of ECs from the basal membrane in the aorta as well as in renal glomeruli. In control littermates, which also received vitamin E-depleted diet, only single swollen mitochondria (heterozygous as well as wild-type mice) as well as few myelinosomes within ECs (heterozygous animals) were detected. As proper endothelial function is crucial for the maintenance of vascular homeostasis, we further analyzed animals receiving the vitamin E-depleted diet for hemodynamic parameters and microvascular function. Indeed, Gpx4iECKO mice displayed an elevated resting arteriolar vessel tone, which was associated with a significantly higher mean arterial blood pressure. These observations suggest that the combined deficiency of Gpx4 and vitamin E in ECs may decrease endothelial vasodilators and limit endothelial function, which in turn could contribute to the thromboembolic events. In contrast, rats deprived of dietary vitamin E have been shown to exhibit a decrease in the relaxation response to acetylcholine as well as elevated MDA serum levels.35 However, species-specific differences as well as differences in the composition and duration of the diet (35 weeks instead of 6 weeks) might account for these variations.

In view of the fact that dying ECs are well known to contribute to a prothrombotic state,28 we paid special attention to signs of thrombotic events. The first indication for thrombus formation came from mice suffering either from hindlimb or forelimb paralysis which was substantiated by autopsy analyses. Two mice revealed macroscopically visible infarct areas either in liver or myocardium. A detailed analysis of different tissues and organs unveiled platelet aggregation, and thus thrombus formation in renal, spleen as well as liver tissue, myocardium and/or spinal cord. Macro- and microinfarctions as well as micro-bleedings were also evident in these organs.

Conclusively, endothelial deletion of Gpx4 along with dietary vitamin E depletion highly increases the risk of life-threatening thrombus formation in mice. Interestingly, even though oxidative stress is a central cause of endothelial dysfunction, data from vitamin E supplementation studies in patients do not reveal a uniform picture. A number of large-scale randomized trials have been disappointing regarding the prevention of cancer or of major cardiovascular events by vitamin E supplementation.36-39 However, one trial, the Women’s Health Study, addressed whether vitamin E supplementation for 10 years may decrease the risk of cardiovascular disease. It is noteworthy that vitamin E supplementation reduced the risk of venous thromboembolism (VTE), especially for women with a prior history of VTE or genetic predisposition.40 Our data provides conclusive evidence that vitamin E acts as a highly efficient back-up system in the prevention of lipid peroxidation processes when other systems are impaired.
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DISCLOSURES
None.

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FIGURE LEGENDS

Figure 1. Generation and Characterization of Gpx4iECKO mice. A Endothelium-specific Gpx4 knockout strategy. Outline of the generation of the conditional Gpx4 knockout allele (top) and breeding scheme used to generate Tamoxifen-inducible endothelial-specific Gpx4 knockout mice (Gpx4iECKO). B Genotyping of tail DNA biopsies. A band of 400 bp indicates the presence of the Cre transgene. A 180-bp band indicates the wild-type allele and the 240-bp band corresponds to the floxed Gpx4 allele. C Mouse lung endothelial cells were isolated from Tamoxifen-treated Gpx4iECKO mice and control littermates (Gpx4control). Successful deletion of Gpx4 was verified by immunoblotting. D,E No difference in vascular density in heart and kidney tissue was observed between Gpx4iECKO and Gpx4control mice (n=5). Analysis was performed by immunofluorescence staining with the endothelial cell marker CD31 (D) and by quantification of the CD31-positive area (E). Data are represented as mean ± SEM. n.s. not significant. Scale bars 50 µm.

Figure 2. Endothelial loss of Gpx4 does not affect tumor growth or tumor-derived angiogenesis. A,B Subcutaneous tumor cell implantation in Gpx4iECKO (n=10) or Gpx4control mice (n=10) results in tumors of similar mass (A) and vascularization (B). Data are represented as mean ± SEM. n.s. not significant, Scale bars 50 µm.

Figure 3. Aortic explants derived from Gpx4iECKO mice show impaired branching. A-D Aortic ring explants from Gpx4iECKO mice (n=5) have similar numbers of sprouts compared to Gpx4control mice (n=5) (A,B). However, the number of branches (C) as well as the overall length of sprouts are significantly decreased (D). Data are represented as mean ± SEM. n.s. not significant, * p < 0.05. Scale bars 200 µm.
Figure 4. Vitamin E-depleted diet results in sudden death of Gpx4\textsuperscript{ECKO} mice. Approximately 80% of Gpx4\textsuperscript{ECKO} mice either die or suffer from paralysis when maintained on a vitamin E-depleted diet.

Figure 5. Thrombus formation in Gpx4\textsuperscript{ECKO} mice receiving a vitamin E-deprived diet. Endothelial cell death (left panel: endothelial cells are detected by CD31 immunofluorescence (red color), TUNEL-positive cells are marked in green) in heart (top panel) and renal (bottom panel) tissue results in thrombus formation (middle panels). CD41 immunofluorescence (green) stains aggregated platelets within different vascular beds (as marked by CD31 immunofluorescence staining in red). Thrombus formation results in multiple pathological conditions such as myocardial infarction (top, right, indicated by arrows) or renal infarction (bottom, right, outlined). Scale bars 50 µm.

Figure 6. Spinal nerve degeneration in Gpx4\textsuperscript{ECKO} mice maintained on a vitamin E-deprived diet. A-C Histological analysis of the spinal cord and the spinal nerves of paralyzed Gpx4\textsuperscript{ECKO} mice revealed micro-bleedings (marked by a dotted line) within the spinal cord (A) as well as spinal nerve degeneration (B). Intravascular thrombus formation (insert in B, marked by an arrow) was only observed in Gpx4\textsuperscript{ECKO} and not in Gpx4\textsuperscript{control} mice (C). D Serum levels of MDA were not elevated Gpx4\textsuperscript{ECKO} mice (n=6) compared to wildtype littermates (n=6) on standard diet. However, when mice received a vitamin E-deprived diet MDA serum levels of Gpx4\textsuperscript{ECKO} mice (n=6) were significantly higher than those of Gpx4\textsuperscript{control} mice (n=7). n.s. not significant, * p < 0.05. Scale bars 20 µm.

Figure 7. Detachment of endothelial cells in Gpx4\textsuperscript{ECKO} mice maintained on a vitamin E-deprived diet. Analysis of the aorta (top) and of renal glomeruli (bottom) by transmission electron microscopy revealed a partial detachment of endothelial cells from the basement membrane (indicated by arrows, right panels) in Gpx4\textsuperscript{ECKO} mice maintained on a vitamin E-depleted diet (n=3), but not in control littermates (n=4). Scale bars 3 µm.

| Table 1 |
|-----------------|-----------------|-----------------|-----------------|-----------------|
| **Hemodynamic parameters** | Gpx4\textsuperscript{control} | Gpx4\textsuperscript{ECKO} | | |
| Number of animals | 6 | 6 | | |
| Mean arterial blood pressure (mmHg) | 69 ± 3 | 79 ± 2 * | | |
| Heart rate (bpm) | 218 ± 35 | 226 ± 13 * | | |
| **Microvascular parameters** | Gpx4\textsuperscript{control} | Gpx4\textsuperscript{ECKO} | | |
| Number of animals | 7 | 6 | | |
| Number of vessels | 76 | 79 | | |
| Maximal vessel diameter (µm) | 38 ± 11 | 35 ± 11 | | |
| Normalized resting diameter (resting diameter/maximal diameter) | 0,89 ± 0,02 | 0,78+/-0,21 * | | |
| Normalized diameter after Norepinephrine (10 µmol/L) (diameter after Norepinephrine/maximal diameter) | 0,47 ± 0,02 | 0,48 ± 0,02 | | |
| Acetylcholine (1 µmol/L) induced vasodilation (% of maximal dilation) | 17± 4 | 24 ± 5 | | |
| Acetylcholine (10 µmol/L) induced vasodilation (% of maximal dilation) | 75± 3 | 83 ± 3 | | |

* * P<0.05
Novelty and Significance

What Is Known?

- It is currently believed that lipid peroxidation contributes to the pathogenesis of cardiovascular diseases.
- Glutathione peroxidase 4 (Gpx4) protects cells from detrimental effects of lipid peroxidation and tissue degeneration.
- Gpx4 controls lipid peroxidation and caspase-independent cell death, both of which can be prevented by vitamin E supplementation in vitro.

What New Information Does This Article Contribute?

- Inducible knockout of Gpx4 in the endothelium does not cause endothelial dysfunction.
- Lowering vitamin E in mouse diet induces lipid peroxidation in endothelial cells leading to detachment of the basement membrane and endothelial cell death in Gpx4 knockout mice.
- Endothelial cell death secondary to Gpx4 loss and vitamin E deprivation causes thromboembolic events, multiple micro-infarctions and early death of Gpx4 knockout mice, establishing a close link between vitamin E and proper Gpx4 function in vivo.

Uncontrolled oxidative degradation of lipids and lipid peroxidation could lead to tissue injury and cell death. The selenoenzyme glutathione peroxidase 4 (Gpx4) reduces lipid hydroperoxides, thus preventing lipid peroxidation. Previous knockout studies in mice have corroborated the importance of Gpx4 in embryogenesis, neuroprotection, retinaprotection, hair follicle morphogenesis, and male fertility. Moreover, the inducible knockout of Gpx4 in fibroblasts induces massive lipid peroxidation and caspase-independent cell death, which can be rescued by the lipophilic antioxidant Vitamin E. Nevertheless, a synergistic effect of vitamin E and Gpx4 has not been rigorously addressed in vivo. Here we show that the inducible loss of Gpx4 in the endothelium does not cause overt endothelial dysfunction. However, when dietary intake of vitamin E was lowered the endothelium-specific Gpx4 knockout mice developed progressive endothelial dysfunction and endothelial cell death, which in turn caused thromboembolic events, microinfarctions and death. These studies establish synergistic actions of vitamin E and selenium-dependent Gpx4, and imply that proper Gpx4 expression and function, along with adequate vitamin E availability, are essential for proper endothelial physiology.
Figure 1. Generation and Characterization of Gpx4^{ECKO} mice

A Endothelium-specific Gpx4 knockout strategy. Outline of the generation of the conditional Gpx4 knockout allele (top) and breeding scheme used to generate Tamoxifen-inducible endothelial-specific Gpx4 knockout mice (Gpx4^{ECKO}).

B Genotyping of tail DNA biopsies. A band of 400 bp indicates the presence of the Cre transgene. A 180-bp band indicates the wild-type allele and the 240-bp band corresponds to the floxed Gpx4 allele.

C Mouse lung endothelial cells were isolated from Tamoxifen-treated Gpx4^{ECKO} mice and control littermates (Gpx4^{control}). Successful deletion of Gpx4 was verified by immunoblotting.

D,E No difference in vascular density in heart and kidney tissue was observed between Gpx4^{ECKO} and Gpx4^{control} mice (n=5). Analysis was performed by immunofluorescence staining with the endothelial cell marker CD31 (D) and by quantification of the CD31-positive area (E). Data are represented as mean ± SEM. n.s. not significant. Scale bars 50 μm.
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Figure 4

Figure 4. Vitamin E-depleted diet results in sudden death of Gpx4<sup>ECKO</sup> mice
Approximately 80% of Gpx4<sup>ECKO</sup> mice either die or suffer from paralysis when maintained on a vitamin E-depleted diet.
Figure 5. Thrombus formation in Gpx4ΔECKO mice receiving a vitamin E-deprived diet

Endothelial cell death (left panel: endothelial cells are detected by CD31 immunofluorescence (red color), TUNEL-positive cells are marked in green) in heart (top panel) and renal (bottom panel) tissue results in thrombus formation (middle panels). CD41 immunofluorescence (green) stains aggregated platelets within different vascular beds (as marked by CD31 immunofluorescence staining in red). Thrombus formation results in multiple pathological conditions such as myocardial infarction (top, right, indicated by arrows) or renal infarction (bottom, right, outlined). Scale bars 50 μm.
Figure 6

**Figure 6. Spinal nerve degeneration in Gpx4<sup>ECKO</sup> mice maintained on a vitamin E-deprived diet**

A-C Histological analysis of the spinal cord and the spinal nerves of paralyzed Gpx4<sup>ECKO</sup> mice revealed micro-bleedings (marked by a dotted line) within the spinal cord (A) as well as spinal nerve degeneration (B). Intravascular thrombus formation (insert in B, marked by an arrow) was only observed in Gpx4<sup>ECKO</sup> and not in Gpx4<sup>control</sup> mice (C). D Serum levels of MDA were not elevated Gpx4<sup>ECKO</sup> mice (n=6) compared to wildtype littermates (n=6) on standard diet. However, when mice received a vitamin E-deprived diet MDA serum levels of Gpx4<sup>ECKO</sup> mice (n=6) were significantly higher than those of Gpx4<sup>control</sup> mice (n=7). n.s. not significant, *p* < 0.05. Scale bars 20 μm.
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Analysis of the aorta (top) and of renal glomeruli (bottom) by transmission electron microscopy revealed a partial detachment of endothelial cells from the basement membrane (indicated by arrows, right panels) in Gpx4<sup>IECKO</sup> mice maintained on a vitamin E-depleted diet (n=3), but not in control littermates (n=4). Scale bars 3 μm.
Combined Deficiency in Glutathione Peroxidase 4 (Gpx4) and Vitamin E Causes Multi-Organ Thrombus Formation and Early Death in Mice
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SUPPLEMENTAL MATERIAL

Methods

Genotyping

Tail probes were lysed in 250 µl of DirectPCR Tail reagent (#31-101-T, PEQLAB, Erlangen, Germany) supplemented with 0.3 mg/ml Proteinase K (#03115836001, Roche Applied Sciences, Mannheim, Germany) at 55°C under continuous agitation for 12 hours. Subsequently, Proteinase K was inactivated by incubating at 85°C for 45 min after which probes were stored at 4°C before genotyping. The following primers were used: PFforw1 (5′-ACT CCC CGT GGA ACT GTG AGC TTT GTGC-3′), PFrev1 (5′-GTG TAC CAC GTA GGT ACTGCTGC-3′), CreD (5′-CAC GAC CAA GTG ACA GCA ATG CTG -3′) and CreE (5′-CAG GTA GTT ATT CGG ATC ATC AGC-3′).

Isolation of endothelial cells from heart and lung tissue

Two weeks following the last tamoxifen application, ECs isolated from heart and lung tissue were analyzed by western blotting to confirm the loss of endothelial Gpx4 expression. Mice were sacrificed and heart and lungs were immediately explanted. After rinsing in PBS, these organs were cut into pieces of approximately 1 mm³. A single cell suspension was established by incubating these pieces in Collagenase A (0.2 mg/ml in PBS, #10103586001, Roche Diagnostics, Mannheim, Germany) for one hour under continuous agitation. Subsequently, the suspension was centrifuged at 400g for 10 min at 4°C. The pellet was diluted in ice cold Endothelial Cell Growth Medium MV 2 (C-22221, PromoCell, Heidelberg, Germany) and filtered through a 70µm cell strainer. Isolation of ECs was performed using a rat anti-mouse CD31 antibody (1:50, BM 4086, Acris Antibodies GmbH, Herford, Germany), goat anti-rat IgG Micro Beads (20 µl per 10⁷ cell, #130-048-501, Miltenyi Biotec, Bergisch Gladbach, Germany) and MACS MS Columns (#130-042-201, Miltenyi Biotec) according to the manufacturer’s manual.

Immunoblotting

Isolated ECs were lysed in protein lysis buffer (20 mM Tris, 137 mM NaCl, 2 mM EDTA, 10% Glycerol, 0.1% Sodium deoxycholate, pH 7.4) supplemented with a protease inhibitor cocktail (Roche, Mannheim, Germany). Protein quantification was performed using the BCA™ Protein Assay (Perbio, Fisher-Scientific, Schwerte, Germany). Detection of Gpx4 was achieved with a monoclonal peptide antibody specific for Gpx4. The monoclonal rat C-terminal antibody 1B4 was produced as described previously. Each blot was reprobed for actin (1:1000, A2066, Sigma-Aldrich). The appropriate HRP-conjugated secondary antibodies were purchased from Dianova (1:5000, Dianova GmbH, Hamburg, Germany). Visualization was achieved by use of the ECL™ detection reagent (GE Healthcare Europe GmbH, Freiburg, Germany). Semiquantitative analysis was performed using the Wasabi imaging software (Hamamatsu Photonics Deutschland, Herrsching, Germany).

Immunohistochemistry and image analysis

Sections were stained with the following antibodies: CD31 antibody (1:200, BM4086, Acris Antibodies) to label ECs and CD41 (1:200, GTX76007, Acris Antibodies) to detect platelet aggregation. Omission of the primary antibody served as negative control. The following secondary antibodies were used: goat anti-rat Alexa 488-conjugated IgG as well as goat anti-rat Alexa 568-conjugated IgG (1:200, all antibodies were purchased from Molecular Probes, Invitrogen) and biotinylated goat anti-rat IgG (1:200, Dianova, Hamburg, Germany). Slides for peroxidase staining were incubated with Peroxidase-conjugated streptavidin (Vectastain
KIT ABC, Vector Laboratories, Linaris, Wertheim-Bettingen, Germany). Thereafter, slides were incubated with Vector® DAB kit or AEC kit (Vector Laboratories.). Dying cells were stained using the ApopTag Fluorescein In Situ Apoptosis Detection Kit (S 7110, Serologicals Corporation, Millipore GmbH, Schwalbach, Germany) according to the manufacturer’s recommendations.
Sections were counterstained with hematoxylin, and mounted with elvanol. Sections for immunofluorescence were counterstained with DAPI (4,6-diamidino-2-phenylindole). Immunostaining was analyzed using the Olympus BX41 microscope in combination with the digital camera CAMEO C-5050 and the software Olympus DP-Soft v3.2 (Olympus, Tokio, Japan).

**Ex Vivo Mouse Aortic Ring Angiogenesis Assay**

*Ex vivo* angiogenesis was studied by culturing mouse aortic rings in a three-dimensional Matrigel matrix. Thoracic aortas were removed from mice sacrificed by cervical dislocation and immediately transferred into ice-cold PBS. The peri-aortic fibroadipose tissue was carefully removed with microdissecting forceps and iridectomy scissors, paying special attention not to damage the aortic wall. One millimeter long aortic rings were sectioned and extensively rinsed in PBS. These ring-shaped explants were embedded into a 48-well plate pre-coated with 40 µl of a 1:1 mixture of Matrigel (#354234, BD Biosciences, Heidelberg, Germany) and Endothelial Cell Growth Medium MV 2 (C-22022, PromoCell). The aortic rings were covered with 40 µl of the same mixture and incubated at 37°C, 5% O2 and 5% CO2 in Endothelial Cell Growth Medium MV 2 for 14 days. Medium was changed every third day. In a second set of experiments aortic rings of Gpx4iECKO mice (n=5) as well as control littermates (n=6) were cultured in the presence or absence of (±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox; 1 µM, #56510; Sigma-Aldrich), a water-soluble analog of vitamin E.

**Hemodynamic parameters and endothelial function in vivo**

Two weeks following the last tamoxifen application (and 8 weeks after the vitamin E depleted diet was started), but prior to the development of clinical manifestations mice were anesthetized by intraperitoneal application of fentanyl (0.04 mg/kg; CuraMED Pharma GmbH, Karlsruhe, Germany), medetomidine (0.4 mg/kg; Pfizer GmbH, Berlin, Germany), and midazolam (4 mg/kg; Ratiopharm GmbH, Ulm, Germany). A catheter was placed into the left femoral artery, and mean arterial blood pressure (MAP) and heart rate were measured using the PowerLab 16/35 data acquisition system (ADInstruments Germany, Spechbach, Germany). Arteriolar resting tone and endothelial function were investigated in arterioles of the cremaster muscle and calculated as described previously. Due to a relatively large normalized resting diameter in both groups, which was probably due to the anesthesia, norepinephrine (1 µmol/L; Sanofi Aventis Germany, Frankfurt, Germany) was added to the superfusion buffer to normalize vessel tone for the subsequent investigation of acetylcholine-dependent changes in vessel diameter. At the end of each experiment, the maximal vasodilatory capacity of each vessel was determined by combined superfusion with acetylcholine (30 µmol/L), adenosine (30 µmol/L) and A769662 (100 µmol/L), an activator of AMP-activated protein kinase. Measurements of the inner diameter of the vessels were normalized to the maximal possible dilation and expressed as percent of maximal dilation using the formula: (D_{after treatment} - D_{before treatment})/(D_{Max} - D_{before treatment})×100.

**Transmission Electron Microscopy (TEM)**

Tissue was fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer pH 7.4 (Science Services, Munich, Germany), postfixed with 2% osmium tetroxide, dehydrated in gradual ethanol (30-100%) and propylene oxide and embedded in Epon (Merck, Darmstadt,
Semithin sections were stained with toluidine blue. Ultrathin sections of 50 nm were collected on 200 mesh copper grids, stained with uranyl acetate and lead citrate before examination with a Zeiss Libra 120 Plus transmission electron microscope (Carl Zeiss NTS GmbH, Oberkochen, Germany). Pictures were acquired using a Slow Scan CCD-camera and iTEM software (Olympus Soft Imaging Solutions, Münster, Germany).
Online Figure I. Recovery of branching of aortic explants derived from Gpx4iECKO mice by addition of Vitamin E

A-D Addition of Vitamin E is able to reestablish the decreased branching of aortic explants derived from Gpx4iECKO mice (A,B). Vitamin E does not significantly influence the number of sprouts derived from aortic ring explants from WT (n=6) or KO mice (n=5) (C). The significantly reduced overall sprout length of explants from KO mice does not fully recover when Vitamin E is added to the cell culture medium (D). Data are represented as mean ± SEM (A,B) or as mean ± SD (C). n.s. not significant, * p < 0.05. Scale bars 200µm.
Online Figure II. Inducible depletion of Gpx4 in mouse eEPCs leads to massive cell death

A Embryonic endothelial progenitor cells (eEPCs) were isolated from conditional Gpx4 knockout embryos. Cells were transfected with a Tamoxifen (TAM)-inducible Gpx4 knockout system (MERCreMER). B Immunoblot analysis with a monoclonal antibody directed against the murine Gpx4 peptide confirms the deletion of Gpx4 protein expression after TAM administration. C Tamoxifen-induced deletion of Gpx4 in eEPCs results in cell death of Gpx4fl/fl cells which was prevented when Trolox was added to the cell culture medium (n=4). D Addition of Tamoxifen to Gpx4wt/fl cells did not cause cell death (n=4).
Online Figure III. Adaptation to endothelial deletion of Gpx4 resulted in a slightly alleviated phenotype when mice were fed a vitamin E-depleted diet

On a standard diet, endothelial depletion of Gpx4 did not result in any obvious phenotype within the first 6 weeks following knockout induction. Subsequent administration of a vitamin E-depleted diet, however, caused the onset of thrombus-induced pathological conditions in more than half of the Gpx4iECKO mice.