Combined Deficiency in Glutathione Peroxidase 4 and Vitamin E Causes Multiorgan Thrombus Formation and Early Death in Mice


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: Endothelium-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. In stark contrast, aortic explants from endothelium-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. Therefore, mice were fed a vitamin E–depleted diet for 6 weeks before endothelial deletion of Gpx4 was induced by 4-hydroxytamoxifen. Surprisingly, ≈80% of the knockout mice died. Histopathological analysis revealed detachment of endothelial cells from the basement membrane and 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 microinfarctions, and paraplegia.

Conclusions: Here, we show for the first time that in the absence of Gpx4, sufficient vitamin E supplementation is crucial for endothelial viability. (Circ Res. 2013;113:408-417.)

Key Words: α-tocopherol ■ endothelium, vascular ■ oxidant stress ■ thrombosis ■ vascular endothelial function

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), 1 of 8 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 and a wide range of lipid hydroperoxides, including those derived from lipoprotein particles and from cholesterol and cholesteryl esters.4,6 Disruption of Gpx4 in the mouse leads to early embryonic lethality at embryonic day 7.5,13 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 lipooxygenase-derived lipid peroxidation rather than accumulation of water-soluble oxygen radicals. Increased lipid peroxidation causes cell death in a caspase-independent, apoptosis-inducing factor–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). In 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 after knockout induction. 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. Because the ability of cells to upregulate intrinsic antioxidant enzymes is fundamentally important in protecting cells from enhanced oxidative stress, several studies addressed whether overexpression of Gpx4 might also increase the resistance of the cell against high levels of lipid hydroperoxides. Indeed, Gpx4 overexpression in smooth muscle cells lowered oxidized low-density lipoprotein–induced proliferation compared with untransfected smooth muscle cells. In accordance, overexpression of Gpx4 in apolipoprotein E–deficient mice (ApoE−/−) inhibited the development of atherosclerosis by decreasing lipid peroxidation. Remarkably, such mice displayed a diminished number of dying endothelial cells (ECs). Furthermore, high amounts of Gpx4 markedly decreased the sensitivity against hydrogen peroxide–induced cytotoxicity in ECs in vitro. These results indicate the importance of Gpx4 in maintaining EC function and vascular wall integrity and prompted us to study the as-yet unexplored role of this redox enzyme in resting and 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 under baseline conditions or under forced angiogenesis after 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 ECs in vivo, we interbred Gpx4−/− mice 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). These mice were further crossed with Gpx4−/−, Gpx4−/+ and Gpx4+/- mice to generate litters containing Gpx4−/− Cdh5(PAC)-CreERT2 (referred to as Gpx4iECKO) and control littermates (referred to as Gpx4 control; Figure 1A). Control littermates nonstandard abbreviations and acronyms

<table>
<thead>
<tr>
<th>Nonstandard Abbreviations and Acronyms</th>
</tr>
</thead>
<tbody>
<tr>
<td>EC</td>
</tr>
<tr>
<td>eEPC</td>
</tr>
<tr>
<td>Gpx4</td>
</tr>
<tr>
<td>Gpx4&lt;sub&gt;lox/lox&lt;/sub&gt;</td>
</tr>
<tr>
<td>Gpx4&lt;sub&gt;lox/+&lt;/sub&gt;</td>
</tr>
<tr>
<td>MDA</td>
</tr>
<tr>
<td>MEF</td>
</tr>
<tr>
<td>TUNEL</td>
</tr>
</tbody>
</table>

Figure 1. Generation and characterization of Gpx4<sup>lox/lox</sup> 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 (TAM)-inducible endothelium-specific Gpx4 knockout mice (Gpx4<sup>lox/lox</sup>). 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 (wt) allele, and the 240-bp band corresponds to the floxed Gpx4 allele. C, Mouse lung endothelial cells were isolated from tamoxifen-treated Gpx4<sup>lox/lox</sup> mice and control littermates (Gpx4<sup>lox/+</sup>). Successful deletion of Gpx4 was verified by immunoblotting. D and E, No difference in vascular density in heart and kidney tissue was observed between Gpx4<sup>lox/lox</sup> and Gpx4<sup>lox/+</sup> 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 means±SEM. n.s Indicates not significant. Scale bars=50 μm.
include mice of the following genotypes: Gpx4lox/lox, Gpx4fl/fl, and Gpx4fl/fl CDh5(PAC)-CreERT2. To activate Cre recombinase in mice carrying the CDh5(PAC)-CreERT2 transgene, 4-hydroxytamoxifen (TS648, Sigma-Aldrich, Deisenhofen, Germany) was administered by oral gavage on 5 consecutive days followed by a final sixth oral application 1 week thereafter (30 μg tamoxifen per gram body weight per day, diluted in corn oil; CS267, 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 (no. 15791-147 vitamin E-depleted diet [ssniff] containing 7 mg/kg vitamin E as compared with 55 mg/kg in normal chow) for 26 weeks before knockout induction. In a second set of experiments, the order of the events was inverted: First, endothelium-specific Gpx4 deletion was induced. The dietary depletion of vitamin E was initiated 6 weeks later. In both settings, the vitamin E-depleted diet was applied to knockout and 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 ECs From Heart and Lung Tissue

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

Isolation of ECs was performed using a rat antimagne CD31 antibody (1:50; BM 4086, Acris Antibodies GmbH, Herford, Germany), goat antirat IgG Micro Beads (20 μL per 107 cells; No. 130-048-501, Miltenyi Biotec, Bergisch Gladbach, Germany), and MACS MS Columns (No. 130-042-201, Miltenyi Biotec) according to the manufacturer’s manual.

Dedeployment of ECs From Brain Tissue

One hemisphere of the brain (3 Gpx4fl/fl and 3 Gpx4lox/lox mice) was cut into pieces of ≈1 mm3 and homogenized using the Neural Tissue Dissection Kit (130-092-628, Miltenyi Biotec). The single-cell solution was filtered through a 70-μm cell strainer, rinsed 3 times, and centrifuged at 400g for 10 minutes at 4°C. Depletion of the ECs was accomplished using a rat antimagne CD31 antibody (1:50; BM 4086, Acris Antibodies), goat antirat IgG Micro Beads (20 μL per 107 cells; Miltenyi Biotec), and MACS LD columns (No. 130042901, Miltenyi Biotec) according to the 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). Semi-quantitative analysis was performed using the Wasabi imaging software (Hamamatsu Photonics Deutschland, Herrsching, Germany).

Cell Lines and Reagents

Lewis Lung Carcinoma cells were cultured at 37°C under 5% CO2, and 5% O2 in Dulbecco modified Eagle medium (No. 41965-039, Invitrogen, Karlsruhe, Germany), supplemented with 10% fetal bovine serum (S 0115 Biochrom, Berlin, Germany), 2 mmol/L L-glutamine (No.25030032, Invitrogen), and 100 μU/mL penicillin/streptomycin (No. 15140-122, Invitrogen).

Histology

Five-micrometer sections of 4% (wt/vol, in PBS) paraformaldehyde-fixed and paraffin-embedded material were stained with hematoxylin-eosin as described or with the Masson-Goldner staining kit (No. 100485, Merck Millipore, Billerica, MA) according to the manufacturer’s instructions.

Immunohistochemistry and Image Analysis

Organ samples and tumor tissue samples were snap-frozen in liquid nitrogen and stored at −80°C. Immunohistochemistry and immunofluorescence were performed as described previously. Immunoanalysis was analyzed using the Olympus BX41 microscope in combination with the Camedia C-5050 digital camera and Olympus DP-Soft software version 3.2 (Olympus, Tokyo, Japan).

Ex Vivo Mouse Aortic Ring Angiogenesis Assay

Ex vivo angiogenesis was studied by culturing mouse aortic rings in a 3-dimensional Matrigel matrix. Aortic rings of Gpx4+/- mice and control littermates were cultured in the presence or absence of (-)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox; 1 μmol/L; No. 56510; Sigma-Aldrich), a water-soluble analog of vitamin E.

Embryonic Endothelial Progenitor Cells

To gain further insight into the role of Gpx4 in the endothelial compartment, we used embryonic endothelial progenitor cells (eEPCs), which were isolated from Gpx4fl/fl and Gpx4lox/lox mouse embryos at embryonic day 7.5. These cells were stably transduced with tamoxifen-inducible MERCReMER (MER indicates mutated estrogen receptor) as described.

To maintain the viability of cells after inducing the deletion of Gpx4 by tamoxifen treatment, cells were cultured in the presence of 1 μmol/L 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 Aqual software as described before. After generation of a binary image, the following semiautomatic 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.

Determination of Serum Malondialdehyde Levels by High-Performance Liquid Chromatography

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 minutes followed by centrifugation at 3000g for 10 minutes at 4°C. The serum was collected, and high-performance liquid chromatography analysis was performed as described previously.

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 the Student’s t test or ANOVA followed by Bonferroni correction. Non-Gaussian-distributed data were analyzed by the nonparametric Kruskal-Wallis test for nonpaired data. Values of P<0.05 were considered significant.

Results

Generation and Histopathological Analysis of Gpx4+/- Mice

The breeding strategy for the generation and genotyping of Gpx4+/- mice is depicted in Figure 1A and 1B. Two weeks after the last tamoxifen application, endothelial loss of Gpx4...
expression was verified in ECs isolated from either heart or lung tissue by Western blotting (Figure 1C). Semiquantitative analysis of endothelial Gpx4 expression revealed a 75% reduction in Gpx4ECKO (100% in control mice versus 24.8±4.0% in Gpx4ECKO mice; endothelial Gpx4 expression of control mice was arbitrarily set to 100%). In contrast, after the depletion of ECs from brain tissue, no difference in Gpx4 expression was detectable between Gpx4ECKO and control mice by Western blot analysis, indicating that nonendothelial 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 6 months (the maximum observation period) after knockout induction. Survival rate was analyzed for 15 Gpx4ECKO mice and 21 control mice. The control group involved the following genotypes: Gpx4ECKOlox/lox (10 animals), Gpx4ECKOlodb (2 animals), and Gpx4ECKO Cdh5(PAC)-CreERT2 (9 animals). Immunohistological analysis of the vasculature of various organs (eg, heart, liver, lung, spleen, brain, and kidney) did not reveal any morphological abnormalities in response to endothelial Gpx4 deletion such as vessel density and vessel integrity (exemplarily shown for heart and renal tissue in Figure 1D and 1E).

Tumor Growth and Angiogenesis Are Not Hindered in Gpx4ECKO Mice

Because 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 Lewis Lung Carcinoma cells tumor cells into Gpx4ECKO and 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 in either vascular density (Figure 2B) or 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). Although the decrease in the number of sprouts from aortic explants of Gpx4ECKO mice as compared with controls was not statistically significant (Figure 3B), a significant reduction in both the number of branches (Figure 3C) and 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 with nontreated explants to a significant extent (Online Figure IA and IB). The increase in the overall sprout length observed on Trolox treatment did, however, not reach statistical significance (Online Figure ID).

Inducible Deletion of Gpx4 in Mouse eEPCs Leads to Massive Cell Death

We have shown previously that disruption of the Gpx4 gene in MEFS and in c-myc/ha-ras–transformed MEFS caused rapid cell death.9,10 As a more relevant cellular model for ECs, we generated a tamoxifen-inducible knockout cell system using eEPCs harboring 1 or 2 loxP-flanked Gpx4 alleles (Online Figure IID). These cells were subsequently transfected with MerCreMer, allowing the tamoxifen-dependent inducible deletion of Gpx4. Tamoxifen treatment induced cell death in Gpx4lox/lox but not in Gpx4lov/lov 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 endothelium-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. 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 (7 mg/kg vitamin E) for ≥26 weeks before deletion of endothelial Gpx4. Strikingly, 80% of the Gpx4ECKO mice maintained on this vitamin E–deprived diet died within 3 weeks or suffered from severe paralysis (3 of 18) after the final tamoxifen application (Figure 4) and had to be euthanized.

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 endothelium-specific deletion of \textit{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 1 sudden death was observed in the group of \textit{Gpx4iECKO} mice (10 animals). Five other mice had to be euthanized because their general condition was poor. One of these mice suffered from myocardial infarction; 1 mouse suffered from hemiparesis; and another showed forelimb paralysis. The 2 remaining mice demonstrated multiple microthrombotic events in the kidneys. In the control group, 1 animal (\textit{Gpx4lox/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: \textit{Gpx4lox/lox} (10 animals), \textit{Gpx4lox/loxCdh5(PAC)-CreERT2} (1 animal), and \textit{Gpx4lox/loxCdh5(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 2 crucially important points: First, there is no other antioxidant system than vitamin E that might take over the function of Gpx4 after adaptation to Gpx4 deletion, and second, Cre toxicity could be definitively ruled out as critical culprit of the phenotype.

**Vitamin E–Depleted Diet Increases the Risk of Thrombosis in \textit{Gpx4iECKO} Mice**

Because of paraplegia of some mice (2 of 18 mice suffered from hindlimb paralysis, 1 of 18 from forelimb paralysis), we...

---

**Figure 3.** Aortic explants derived from \textit{Gpx4iECKO} mice show impaired branching. \textit{A} through \textit{D}, Aortic ring explants from \textit{Gpx4iECKO} mice (n=5) have similar numbers of sprouts compared with \textit{Gpx4control} mice (n=5; \textit{A} and \textit{B}). However, the number of branches (\textit{C}) and the overall length of sprouts are significantly decreased (\textit{D}). Data are represented as means±SEM. \textit{n.s} indicates not significant. Scale bars=200 μm. *\textit{P}<0.05.

**Figure 4.** Vitamin E-depleted diet results in sudden death of \textit{Gpx4iECKO} mice. Approximately 80% of \textit{Gpx4iECKO} mice either die or suffer from paralysis when maintained on a vitamin E-depleted diet.
hypothesized that thrombus formation might be one of the underlying reasons for paraplegia and sudden death in Gpx4iECKO mice. Indeed, a systemic histological analysis of different organs confirmed our assumption (Figure 5). One possible reason for the thromboembolic events in Gpx4iECKO mice might be EC death because dying ECs are known to be procoagulant. Therefore, we analyzed sections from multiple organs by combined CD31/terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) staining. In fact, single TUNEL-positive ECs were detectable in lung, kidney, liver, and heart tissue of Gpx4iECKO mice (exemplarily shown for heart and renal tissue in Figure 5; left column, top and bottom), which was in sharp contrast to control littermates in which no TUNEL-positive ECs 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). Such thrombus formation resulted in multiple pathologies like myocardial infarction (Figure 6B) or microinfarctions in the kidney (Figure 5; right column, top and bottom). Microinfarctions and microbleedings could also be detected in various organs and tissues such as spleen, liver, and others (not shown). Intriguingly, paralyzed mice had microbleedings with various organs and tissues such as spleen, liver, and others (not shown).

Mean Arterial Blood Pressure 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, mean arterial blood pressure and heart rates were determined. Mean arterial blood pressure was significantly elevated in Gpx4iECKO mice compared with control mice (79±2 vs. 69±3 mmHg, respectively; n=6 each; P<0.05). In contrast, heart rate was not different between Gpx4iECKO mice and control mice (226±13 versus 218±35 bpm, respectively; Table).

Resting Arteriolar Tone and Acetylcholine-Induced Dilation
A total of 76 arterioles from 7 control mice and 79 arterioles from 6 Gpx4iECKO mice were investigated. Maximal diameters of the arterioles were not significantly different between control littermates (38 μm; range, 18–69 μm) and Gpx4iECKO mice (35 μm; range, 16–70 μm). The arterioles in untreated preparations showed varying normalized diameters (from 0.25–1.00); however, normalized diameter was lower in Gpx4iECKO mice compared with control littermates (0.78±0.02 versus 0.89±0.02; P<0.05), corresponding to an increased arteriolar resting tone. In contrast, acetylcholine-dependent vasodilation (expressed as percent of maximal vessel dilation) as an indicator of endothelial function was not significantly different in Gpx4iECKO mice compared with control mice either in response to low acetylcholine (1 μmol/L; 24±5% versus 17±4% in control mice) or in response to high acetylcholine concentrations (10 μmol/L; 83±3% versus 75±3% in control mice). Correspondingly, the response to 1 μmol/L norepinephrine (which was used to normalize the vessel tone for the investigation of acetylcholine-induced vasodilation) was similar in Gpx4iECKO and control littermates (normalized vessel diameter after norepinephrine, 0.48±0.02 in Gpx4iECKO mice versus 0.47±0.02 in controls). Results are shown in the Table.

Increased Lipid Peroxidation in Vitamin E–Deprived Gpx4iECKO Mice
Malondialdehyde (MDA) is one of the most established indicators of lipid peroxidation. MDA determinations by high-performance liquid chromatography 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 Gpx4iECKO littermates (n=6; 13.06±2.25 versus 11.71±2.14 μmol/L). However, when mice received a vitamin E–depleted diet, significantly higher MDA plasma levels were observed in Gpx4iECKO mice (14.7±3.07 in control mice versus 20.04±2.87 μmol/L in Gpx4iECKO mice; Figure 6D).
mitochondria in renal ECs seemed swollen.

obvious phenotype (Figure 7, left column) except that single
that were also kept on a vitamin E–depleted diet revealed no
E–depleted diet (Figure

µ

(n=7). ns indicates not significant. Scale bars=20

Gpx4iECKO

of paralyzed
Histological analysis of the spinal cord and the spinal nerves
through

C

mantained on a vitamin E–deprived diet. A

Gpx4control

Vitamin E Depletion Leads to EC Ablation in

Gpx4iECKO

Mice

Analysis of the aorta and renal glomeruli by transmission
electron microscopy revealed partial ablation of ECs from
the basement membrane in

Gpx4iECKO

mice (n=6) compared with wild-
type littermates (n=6) on standard diet. However, when mice
received a vitamin E–deprived diet, MDA serum levels of
Gpx4iECKO

mice (n=6) were significantly higher than those of
Gpx4control

mice (n=7). ns indicates not significant. Scale bars=20 µm. *P<0.05.

Figure 6. Spinal nerve degeneration in

Gpx4iECKO

tumor-induced angiogenesis in

Gpx4iECKO

ed when Gpx4 alone was deleted in the endothelium. Similarly,
tumor-induced angiogenesis in

Gpx4iECKO

mice was compara-
tible to that in wild-type littermates, indicating an efficient
backup system under in vivo conditions. Two observations led
us to the assumption that the backup system might be available
only in vivo, not in ex vivo, cell systems: First, Gpx4 knockout eEPCs rap-
display significantly impaired angiogenesis in an ex vivo aortic
ring angiogenesis assay; second, Gpx4 knockout eEPCs rapidly
die when explanted in culture. Indeed, although vitamin
E is not a component of the EC growth medium, the applied
mouse diet is highly supplemented with vitamin E. To substan-
tiate our hypothesis that vitamin E in the diet is masking the
 genetic loss of Gpx4 in vascular ECs in vivo, mice were fed
a vitamin E–depleted diet for ≥6 weeks before the 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; ≥80% of the Gpx4iECKO

mice either suffered
from paralysis (3 of 18) or suddenly died within 3 weeks after
the final tamoxifen application (14 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 activ-
ity, 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 6 weeks before the vitamin
E–depleted diet was initiated. With 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 of
10). The confirmation of the phenotype in the reciprocal proto-
col provided definitive proof for the backup function of vitamin
E for Gpx4 deficiency in ECs in vivo and excluded Cre toxic-
ity as a putative culprit in provoking the observed phenotype.

Vitamin E Depletion Leads to EC Ablation in

Gpx4iECKO

Mice

Analysis of the aorta and renal glomeruli by transmission
electron microscopy revealed partial ablation of ECs from
the basement membrane in

Gpx4iECKO

mice kept on a vitamin
E–deprived diet (Figure 7, right column). Control littermates
that were also kept on a vitamin E–deprived diet revealed no
obvious phenotype (Figure 7, left column) except that single
mitochondria in renal ECs seemed swollen.

Discussion

The endothelium maintains vascular homeostasis through
multiple complex interactions with cells in the vessel wall and
vessel lumen.30 Considerable evidence suggests that oxidative
stress is an important contributing factor in endothelial
dysfunction.31 The functional role of endothelium-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 and its interplay with vitamin E in resting and proliferat-
ing ECs in vivo. Interestingly, vascular integrity was not affect-
ed when Gpx4 alone was deleted in the endothelium. Similarly,
tumor-induced angiogenesis in

Gpx4iECKO

mice was compara-
tible to that in wild-type littermates, indicating an efficient
backup system under in vivo conditions. Two observations led
us to the assumption that the backup system might be available
only in vivo, not in ex vivo, cell systems: First, Gpx4 knockout eEPCs rap-
display significantly impaired angiogenesis in an ex vivo aortic
ring angiogenesis assay; second, Gpx4 knockout eEPCs rapidly
die when explanted in culture. Indeed, although vitamin
E is not a component of the EC growth medium, the applied
mouse diet is highly supplemented with vitamin E. To substan-
tiate our hypothesis that vitamin E in the diet is masking the
 genetic loss of Gpx4 in vascular ECs in vivo, mice were fed
a vitamin E–depleted diet for ≥6 weeks before the 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; ≥80% of the Gpx4iECKO

mice either suffered
from paralysis (3 of 18) or suddenly died within 3 weeks after
the final tamoxifen application (14 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 activ-
ity, 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 6 weeks before the vitamin
E–depleted diet was initiated. With 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 of
10). The confirmation of the phenotype in the reciprocal proto-
col provided definitive proof for the backup function of vitamin
E for Gpx4 deficiency in ECs in vivo and excluded Cre toxic-
ity as a putative culprit in provoking the observed phenotype.

Table. Hemodynamic Parameters and Microvascular Function

<table>
<thead>
<tr>
<th>Gpx4iECKO</th>
<th>Gpx4control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Animals, n</td>
<td>6</td>
</tr>
<tr>
<td>Mean arterial blood pressure, mm Hg</td>
<td>69±3</td>
</tr>
<tr>
<td>Heart rate, bpm</td>
<td>218±35</td>
</tr>
<tr>
<td>Microvascular parameters</td>
<td></td>
</tr>
<tr>
<td>Animals, n</td>
<td>7</td>
</tr>
<tr>
<td>Vessels, n</td>
<td>76</td>
</tr>
<tr>
<td>Maximal vessel diameter, µm</td>
<td>38±11</td>
</tr>
<tr>
<td>Normalized resting diameter (resting diameter/ maximal diameter)</td>
<td>0.89±0.02 0.78±0.21*</td>
</tr>
<tr>
<td>Normalized diameter after norepinephrine (1 µmol/L; diameter after norepinephrine/maximal diameter)</td>
<td>0.47±0.02 0.48±0.02</td>
</tr>
<tr>
<td>Acetylcholine (1 µmol/L)–induced vasodilation, % of maximal dilation</td>
<td>17±4</td>
</tr>
<tr>
<td>Acetylcholine (10 µmol/L) induced vasodilation, % of maximal dilation</td>
<td>75±3</td>
</tr>
<tr>
<td>*P&lt;0.05.</td>
<td></td>
</tr>
</tbody>
</table>
Nevertheless, because there was a severity difference between the 2 groups, we cannot absolutely exclude that Cre activation exacerbated the phenotype. Because 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 MDA, 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 (n=4). Scale bars=3 μm.

![Figure 7. Detachment of endothelial cells (ECs) in Gpx4iECKO mice maintained on a vitamin E–depleted diet](image)

Conclusively, endothelial deletion of Gpx4 and dietary vitamin E depletion highly increase the risk of life-threatening thrombus formation in mice. Interestingly, although 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 concerning the prevention of cancer or of major cardiovascular events by vitamin E supplementation.36–39 However, 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, especially for women with a previous history of venous thromboembolism or genetic predisposition.30 Our data provide conclusive evidence that vitamin E acts as a highly efficient backup system in the prevention of lipid peroxidation processes when other systems are impaired.

Acknowledgments
We thank Heidi Förster, Uta Mamrak, Dorothee Gössel, and Matthias Semisch for excellent technical assistance. We thank Julia Kirsch for his careful reading of the article and his detailed comments and suggestions for further improvement.

Sources of Funding
This work was supported by the Deutsche Forschungsgemeinschaft (DFG) priority Program SPP 1190 to H. Beck and M. Conrad, the Friedrich-Baur Stiftung to H. Beck, the FoFoLe program of the Ludwig-Maximilians University Munich to H. Beck, and a DFG grant (CO 291/2–3) to M. Conrad.

Disclosures
None.

References


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 cause thromboembolic events, multiple microinfarctions, and early death of Gpx4 knockout mice, establishing a close link between vitamin E and proper Gpx4 function in vivo.

Novelty and Significance

Uncontrolled oxidative degradation of lipids and lipid peroxidation could lead to tissue injury and cell death. The selenoenzyme Gpx4 reduces lipid hydroperoxides, thus preventing lipid peroxidation. Previous knockout studies in mice have corroborated the importance of Gpx4 in embryogenesis, neuroprotection, retina protection, 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.
Combined Deficiency in Glutathione Peroxidase 4 and Vitamin E Causes Multiorgan Thrombus Formation and Early Death in Mice
Markus Wortmann, Manuela Schneider, Joachim Pircher, Juliane Hellfritsch, Michaela Aichler, Naidu Vegi, Pirkko Kölle, Peter Kuhlencordt, Axel Walch, Ulrich Pohl, Georg W. Bornkamm, Marcus Conrad and Heike Beck

Circ Res. 2013;113:408-417; originally published online June 14, 2013; doi: 10.1161/CIRCRESAHA.113.279984
Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2013 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/113/4/408
Data Supplement (unedited) at:
http://circres.ahajournals.org/content/suppl/2013/06/14/CIRCRESAHA.113.279984.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation Research 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 Circulation Research is online at:
http://circres.ahajournals.org/subscriptions/
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 ACAGTCTGC-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 CAMEOIA 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.23 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: \[
\left(\frac{D_{\text{after treatment}} - D_{\text{before treatment}}}{D_{\text{Max}} - D_{\text{before treatment}}}\right) \times 100
\]

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 Gpx4\textsuperscript{ECKO} mice.