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BASIC SCIENCE

NADPH oxidase 4 protects against development of endothelial dysfunction and atherosclerosis in LDL receptor deficient mice

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Aims Endothelial dysfunction is an early step in the development of atherosclerosis. Increased formation of superoxide anions by NADPH oxidase Nox1, 2, and 5 reduces nitric oxide availability and can promote endothelial dysfunction. In contrast, recent evidence supports a vasoprotective role of H_2O_2 produced by main endothelial isoform Nox4. Therefore, we analysed the impact of genetic deletion of Nox4 on endothelial dysfunction and atherosclerosis in the low-density lipoprotein receptor (Ldlr) knockout model.

Methods and results *Ex vivo* analysis of endothelial function by Mulvany myograph showed impaired endothelial function in thoracic aorta of $Nox4^{-/-}/Ldlr^{-/-}$ mice. Further progression of endothelial dysfunction due to high-fat diet increased atherosclerotic plaque burden and galectin-3 staining in $Nox4^{-/-}/Ldlr^{-/-}$ mice compared with $Ldlr^{-/-}$ mice. Under physiological conditions, loss of Nox4 does not influence aortic vascular function. In this setting, loss of Nox4-derived H_2O_2 production could be partially compensated for by nNOS upregulation. Using an innovative optical coherence tomography approach, we were able to analyse endothelial function by flow-mediated vasodilation in the murine saphenous artery *in vivo*. This new approach revealed an altered flow-mediated dilation in $Nox4^{-/-}$ mice, indicating a role for Nox4 under physiological conditions in peripheral arteries *in vivo*.

Conclusions Nox4 plays an important role in maintaining endothelial function under physiological and pathological conditions. Loss of Nox4-derived H_2O_2 could be partially compensated for by nNOS upregulation, but severe endothelial dysfunction is not reversible. This leads to increased atherosclerosis under atherosclerotic prone conditions.

Keywords NADPH oxidase 4 • Nox4 • Endothelial dysfunction • $Ldlr^{-/-}$ mice • Atherosclerosis • Flow-mediated dilation

Translational perspective

Genetic deletion of the hydrogen peroxide producing NADPH oxidase 4 (Nox4), as shown in the present study, leads to endothelial dysfunction and increased atherosclerosis under pathological conditions. Consequently, endothelial activation of Nox4 may represent a promising novel strategy for preventing endothelial dysfunction and atherosclerosis and its severe clinical complications. This also suggests that in contrast to the deleterious effects of oxidative stress certain reactive oxygen species might mediate beneficial effects in the vessel wall.

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Introduction

Endothelial dysfunction is an early event in atherosclerosis that precedes clinical symptoms and has prognostic value for future cardiovascular events.[1–8] It is characterized by an imbalance between vasodilation and vasoconstriction, inhibition and promotion of proliferation and migration of smooth muscle cells, prevention and stimulation of adhesion of monocytes and aggregation of platelets.[9] Furthermore, increased formation of reactive oxygen species (ROS) such as superoxide anions can contribute to impaired endothelium-dependent vasodilation by decreased bioavailability of nitric oxide (NO).[10,11]

NADPH oxidase (Nox) enzyme complexes are predominant sources of ROS in the vessel wall.[12,13] Isoforms Nox1, Nox2, Nox4, and Nox5 are expressed in the human vasculature.[14] Nox4 is the predominant isoform in endothelial cells[15] and mainly produces H_2O_2, which might explain functional differences to superoxide anion producing Nox isoforms.[16–18]

The role of different Nox isoforms in cardiovascular physiology and pathophysiology is controversial.[11] Increased expression of Nox1 and Nox2 in vascular cells and macrophages has been reported as contributing to atherosclerosis and vascular diseases.[19–21] Patients with genetic Nox2 deficiency show an enhanced endothelium-dependent relaxation.[22] Nox5 can contribute to the oxidative stress in human coronary artery disease as well.[23] Increasing evidence supports mainly beneficial effects of Nox4 in the cardiovascular system, overexpression of Nox4 reduced angiotensin II-induced increase in blood pressure.[24] Mechanistically, H_2O_2 increases endothelial NO release by AKT-dependent (Ser473/Thr308) phosphorylation.[25–28] In addition, H_2O_2 acts as an endothelium-derived hyperpolarizing factor (EDHF) in mice and man leading to vasorelaxation.[29–31] Global Nox4 knockout mice revealed contractile impairment, cardiac hypertrophy, and intestinal fibrosis after chronic overload.[32] In tamoxifen-inducible Nox4 knockout mice, angiotensin II-mediated aortic inflammation, media hypertrophy, and endothelial dysfunction were enhanced.[33] On the other hand, $Nox4^{-/-}$ mice were protected from oxidative stress, blood–brain barrier leakage, and neuronal apoptosis in a stroke model.[34] Cardiac hypertrophy, fibrosis, and apoptosis after pressure overload were reduced in a model of cardiac-specific deletion of Nox4.[35] These data suggest different cell- and dose-dependent effects of Nox4 in cardiovascular diseases.[36]

To investigate the diverse role of Nox4, we analysed the effects of genetic deletion of Nox4 in the development of endothelial dysfunction and atherosclerosis in mice *in vivo*.

Methods

All materials and methods are available in Supplementary material online.

Results

$Nox4^{-/-}/Ldlr^{-/-}$ mice develop endothelial dysfunction

First, we analysed endothelial function in aortas of 10-week-old C57BL/6J (wild type), $Ldlr^{-/-}$, and $Nox4^{-/-}$ mice. We observed no changes in endothelium-dependent relaxation. In contrast, $Nox4^{-/-}/Ldlr^{-/-}$ double knockout mice of the same age developed endothelial dysfunction (*Figure 1A* and *B*). No changes in smooth muscle function were observed between the strains of mice (*Figure 1C*). In addition, endothelial dysfunction could be induced by application of catalase to aortic segments of $Ldlr^{-/-}$ mice (*Figure 1D* and *E*). By blocking the calcium-activated potassium channels with large conductance (BK channels) with paxilline endothelial function of $Ldlr^{-/-}$ mice, but not wild-type mice, was declined to the level of endothelial dysfunction in aortic rings of $Nox4^{-/-}/Ldlr^{-/-}$ mice (*Figure 1F* and *G*).

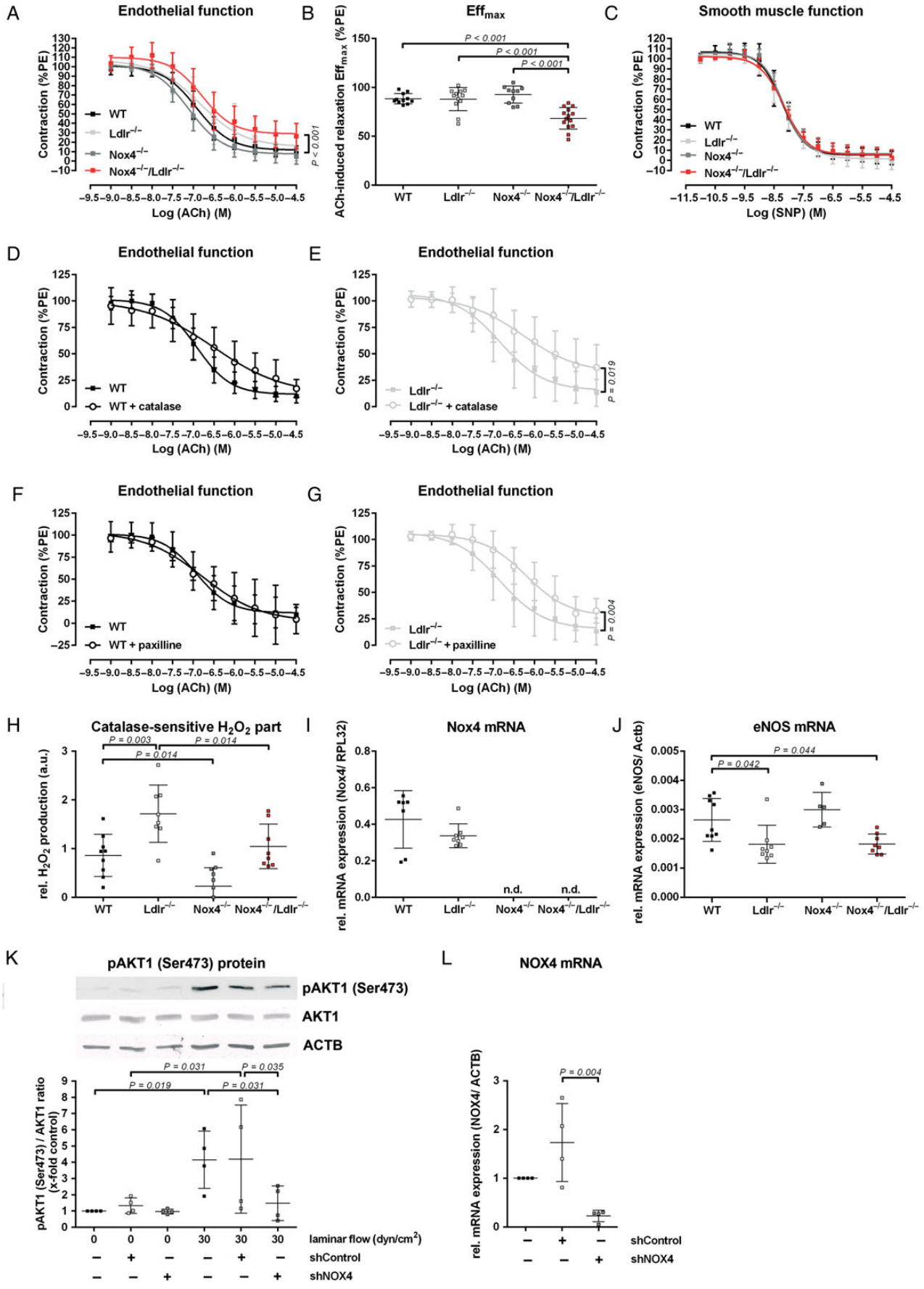
Hydrogen peroxide release of aortas revealed significantly higher levels in $Ldlr^{-/-}$ mice. Aortas of $Nox4^{-/-}/Ldlr^{-/-}$ mice released significantly lower levels of hydrogen peroxide compared with the $Ldlr^{-/-}$ mice (*Figure 1H*). Interestingly, both $Ldlr^{-/-}$ and $Nox4^{-/-}/Ldlr^{-/-}$ mice expressed significant lower eNOS mRNA in the aorta compared with wild-type mice. This suggests that hydrogen peroxide might partially compensate NO as a vasodilator in $Ldlr^{-/-}$ mice (*Figure 1I* and *J*). *In vitro* experiments in human umbilical vein endothelial cells showed activation of pAKT1 (Ser473) by high laminar flow of 30 dyn/cm^2. Downregulation of NOX4 by shRNA diminished pAKT1 (Ser473) phosphorylation (*Figure 1K* and *L*). We assume that hydrogen peroxide derived from NOX4 acts via pAKT1 (Ser473) phosphorylation on eNOS activation. We observed severely increased levels of LDL cholesterol, total cholesterol, and triglyceride in the serum of $Ldlr^{-/-}$ and $Nox4^{-/-}/Ldlr^{-/-}$ mice (see Supplementary material online). Thus, hydrogen peroxide seems to be particularly essential under pathological conditions.

$Nox4^{-/-}/Ldlr^{-/-}$ mice on high-fat diet develop increased atherosclerosis

To determine the outcome of the severe endothelial dysfunction, we investigated $Nox4^{-/-}/Ldlr^{-/-}$ mice after 20 weeks on a high-fat diet. Body weight development, energy intake, blood glucose, serum lipid parameters, weight of white adipose tissue, kidney, heart, and liver did not differ between $Ldlr^{-/-}$ and $Nox4^{-/-}/Ldlr^{-/-}$ mice (see Supplementary material online, *Figure S3* and *Tables S2, S3*). Plaque burden in the aortic arch of $Nox4^{-/-}/Ldlr^{-/-}$ mice was significantly higher, compared with $Ldlr^{-/-}$ mice, as indicated by Elastica van Gieson staining (*Figure 2A*). Similarly, Galectin-3 staining was significantly higher in aortic arch sections of $Nox4^{-/-}/Ldlr^{-/-}$ mice, compared with $Ldlr^{-/-}$ mice, further supporting a more severe atherosclerotic phenotype in the double knockout mice (*Figure 2B*). Collagen content in the media analysed by Sirius Red staining was significantly increased in $Nox4^{-/-}/Ldlr^{-/-}$ mice (*Figure 2C*). Under the severe conditions of LDL receptor knockout, the loss of Nox4 led to a higher progression of atherosclerosis.

Compensation of hydrogen peroxide release in older $Nox4^{-/-}$ mice

The importance of hydrogen peroxide is also supported by the fact that decreased aortic H_2O_2 levels in young $Nox4^{-/-}$ mice were restored to levels of wild-type mice at 26 weeks of age (*Figure 3A* and *B*). This effect could be blocked when incubating aortic segments with NOS blocker L-NAME (*Figure 3B*), indicating a role of nNOS



as potential source of H_2O_2. Similar to the compensation of hydrogen peroxide in the 26-week-old Nox4$^{-/-}$ mice, nNOS mRNA expression was significantly increased in aortas of 26-week-old but not 10-week-old Nox4$^{-/-}$ mice (*Figure 3C* and *D*). Aortic endothelial function of 26-week-old Nox4$^{-/-}$ was not altered (*Figure 3E*). However, NO availability seemed to be decreased in aortic segments of Nox4$^{-/-}$ mice. These mice showed a trend to decreased L-NAME-induced endothelium-dependent constriction (*Figure 3F*).

Saphenous artery of Nox4$^{-/-}$ mice show impaired flow-mediated dilation *in vivo*

Finally, we analysed endothelial function of *Arteria saphena* of 26-week-old wild-type, Nox4$^{-/-}$, and Ldlr$^{-/-}$ mice *in vivo*. We developed a method of determining flow-mediated dilation by optical coherence tomography (OCT). Cross-sectional tomographic images (25 B-scans per second) at resting conditions, after vessel clamp release and after sodium nitroprusside application were analysed (*Figure 4A–C*). In addition, a continuous OCT recording was performed over a 10-min period after vessel clamp release to analyse flow-mediated vasodilation (*Figure 4D*). Analysis showed significant increase in vessel diameter due to flow-mediated dilation in wild-type mice, while no significant increase in vessel diameter after clamp release could be detected in either Nox4$^{-/-}$ or Ldlr$^{-/-}$ mice (*Figure 4C*). Sodium nitroprusside caused similar increases of vessel diameter in mice strains.

Discussion

In the present study, we were able to show that loss of H_2O_2-releasing Nox4 in a genetic background of hypercholesterolaemia leads to severe endothelial dysfunction. The reduced vasodilation capacity in the Nox4$^{-/-}$/Ldlr$^{-/-}$ mice was identical to the declined maximal acetylcholine-induced relaxation of aortic segments of Ldlr$^{-/-}$ mice which were pre-incubated with hydrogen peroxide-degrading catalase or BK_{Ca} channel inhibitor paxilline. This implies a role of H_2O_2 as a vasodilator under pathological conditions. In agreement with this assumption, we did not find changes in vascular function in 10-week-old Nox4$^{-/-}$ mice. It was recently shown that increased H_2O_2 release by overexpression of Nox4 enhances vasodilation in mice.[24] Aortas of Ldlr$^{-/-}$ mice released more H_2O_2 than wild-type mice. Compared with Ldlr$^{-/-}$, Nox4$^{-/-}$/Ldlr$^{-/-}$ double knockout mice showed significantly lower levels of H_2O_2. This indicates a specific role for Nox4-released H_2O_2 in Ldlr$^{-/-}$ mice. In support of this, we found a lower eNOS mRNA expression in Ldlr$^{-/-}$ mice as well as Nox4$^{-/-}$/Ldlr$^{-/-}$ mice, which could explain the importance of H_2O_2 for vasorelaxation in these mice. H_2O_2 might act as an EDHF in mice and man.[29–31] Endothelium-derived hyperpolarizing factors can open calcium-activated potassium channels leading to a hyperpolarized membrane of the vascular smooth muscle cell and subsequent vasorelaxation.[37] Under pathological conditions of atherosclerosis and hypertension H_2O_2 can be involved in compensation of vasorelaxation in large vessels.[27] In mice with DOCA-salt hypertension and uncoupled eNOS, endothelium-derived H_2O_2 compensated to maintain vasodilation.[38] We observed an altered endothelial function in Ldlr$^{-/-}$ mice after application of BK_{Ca} channel inhibitor paxilline. The reduction was comparable with the endothelial dysfunction found in Nox4$^{-/-}$/Ldlr$^{-/-}$ mice. This suggests that H_2O_2 in Ldlr$^{-/-}$ mice leads via opening of potassium channels to hyperpolarization-mediated dilation. Thereby, H_2O_2 compensates the loss of other vasodilator mechanisms. Furthermore, unlike superoxide, H_2O_2 does not further decrease bioavailability of NO. H_2O_2 is also described as increasing expression and activation of AKT/eNOS pathway.[25,26] Our observation, that downregulation of NOX4 leads to less phosphorylation of pAKT1 (Ser473) under laminar shear stress, suggests a protective role of Nox4 generated H_2O_2 via this pathway.

We analysed the consequences of the endothelial dysfunction in Nox4$^{-/-}$/Ldlr$^{-/-}$ mice by feeding a high-fat diet for 20 weeks. Plaque burden was significantly higher in the Nox4$^{-/-}$/Ldlr$^{-/-}$ double knockout compared with Ldlr$^{-/-}$ mice. Inflammation marker Galectin-3 and collagen content was also increased in Nox4$^{-/-}$/Ldlr$^{-/-}$ compared with Ldlr$^{-/-}$ mice. Therefore, we are the first to document a potential protective role of Nox4 in the background of hypercholesterolaemia.

The role of other Nox isoforms in atherosclerosis is controversial. Some studies using knockouts of subunits of the Nox2 complex showed anti-atherosclerotic effects[21,39,40] while others did not.[41] The non-selective Nox1/Nox4 inhibitor GKT137831 slightly reduced atherosclerotic plaque area in diabetic ApoE$^{-/-}$ mice.[42] Furthermore, Nox1 deletion, but not Nox4, decreased vascular adhesion of leukocytes and expression of inflammation markers,[43] indicating that the effects of inhibitor GKT137831 might mainly result from blocking Nox1. Nox4 expression was mainly upregulated

Figure 1 Nox4$^{-/-}$/Ldlr$^{-/-}$ mice develop endothelial dysfunction. (*A*) Concentration–response curve for acetylcholine in aortic segments of 10-week-old wild-type, Ldlr$^{-/-}$, Nox4$^{-/-}$, and Nox4$^{-/-}$/Ldlr$^{-/-}$ mice ($n \geq 10$) precontracted with phenylephrine. (*B*) Maximal effect of 30 μmol/L ACh on aortic segments of wild-type, Ldlr$^{-/-}$, Nox4$^{-/-}$, and Nox4$^{-/-}$/Ldlr$^{-/-}$ mice ($n \geq 10$). (*C*) Concentration–response curves for sodium nitroprusside in aortic segments of wild-type, Ldlr$^{-/-}$, Nox4$^{-/-}$, and Nox4$^{-/-}$/Ldlr$^{-/-}$ mice ($n \geq 7$). (*D* and *E*) Concentration–response curve for acetylcholine in aortic segments of 10-week-old wild-type and Ldlr$^{-/-}$ mice with catalase ($n \geq 9$). (*F* and *G*) Concentration–response curve for acetylcholine in aortic segments of 10-week-old wild-type and Ldlr$^{-/-}$ mice with paxilline ($n \geq 9$). (*H*) Amplex red assay for hydrogen peroxide generation of *Aorta thoracalis* segments of wild-type, Ldlr$^{-/-}$, Nox4$^{-/-}$, and Nox4$^{-/-}$/Ldlr$^{-/-}$. H_2O_2 formation was measured as the catalase-sensitive part of the signal (a.u.: arbitrary units) ($n \geq 8$). (*I* and *J*) Real-time polymerase chain reaction of murine aorta from wild-type, Ldlr$^{-/-}$, Nox4$^{-/-}$, and Nox4$^{-/-}$/Ldlr$^{-/-}$ mice ($n \geq 5$). (*K*) Western blot analysis of pAKT1 (Ser473) in human umbilical vein endothelial cells transduced with shControl or shNOX4 for 48 h. Cells were either kept under static conditions or exposed to laminar shear stress (30 dyn/cm^2) for 5 min (n=4. Statistics: one-way analysis of variance Duncan's method). (*L*) Real-time polymerase chain reaction of NOX4 in human umbilical vein endothelial cells. Cells were transduced for 72 h with shControl or shNOX4 ($n = 4$).

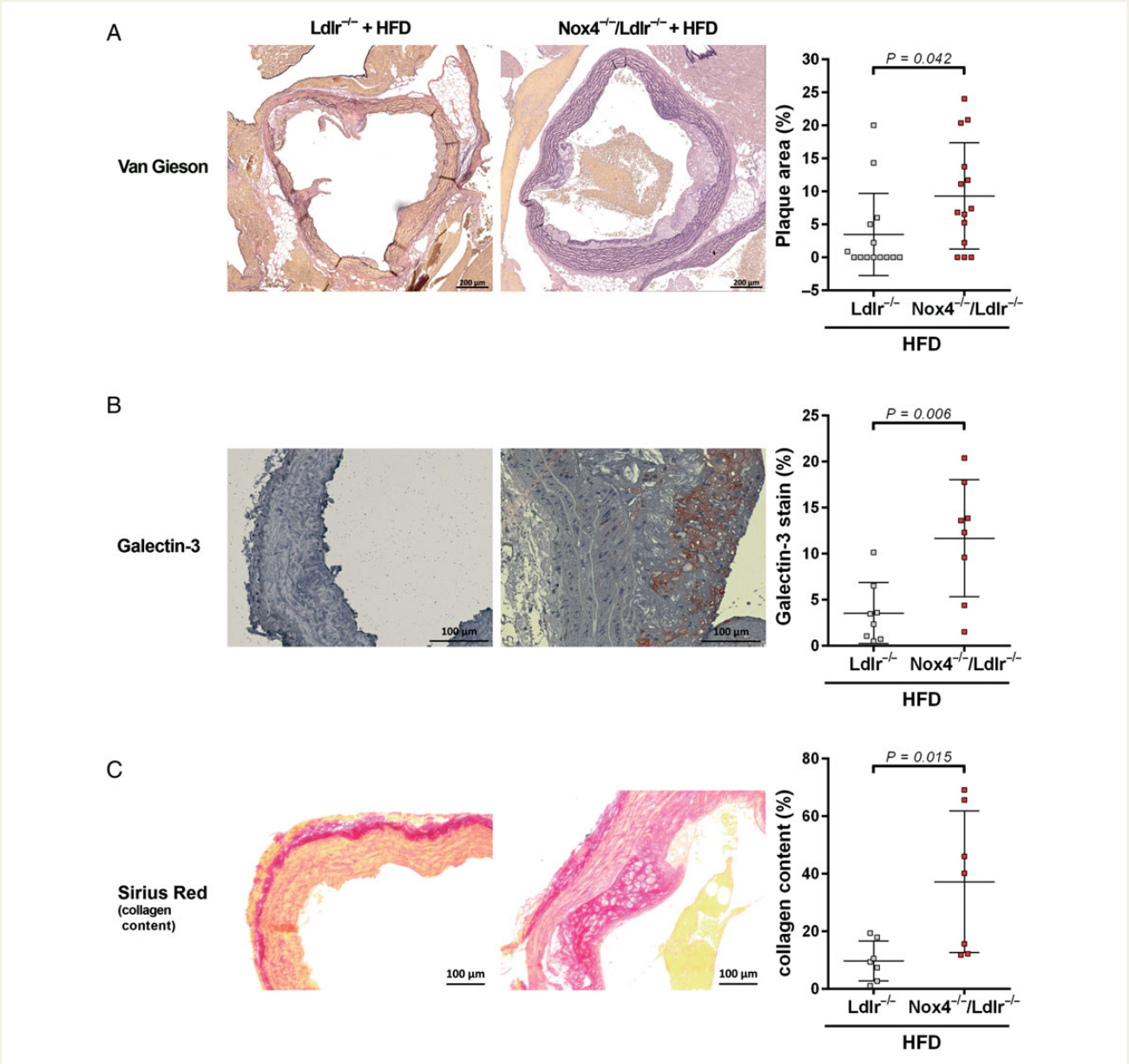


Figure 2 Nox4$^{-/-}$/Ldlr$^{-/-}$ mice develop increased atherosclerosis on high-fat diet. (*A*) Atherosclerotic plaque burden: Plaque area relative to total vessel area of aortic arch sections was shown with Elastica Van Gieson staining from Ldlr$^{-/-}$ and Nox4$^{-/-}$/Ldlr$^{-/-}$ mice after 20 weeks on high-fat diet ($n = 14$). (*B*) Galectin-3 staining of aortic arch sections of Ldlr$^{-/-}$ and Nox4$^{-/-}$/Ldlr$^{-/-}$ mice after 20 weeks on high-fat diet ($n \geq 8$). (*C*) Collagen content: Sirius Red-positive staining of plaque and media of aortic arch sections from Ldlr$^{-/-}$ and Nox4$^{-/-}$/Ldlr$^{-/-}$ mice after 20 weeks on high-fat diet ($n = 7$).

in the atheroma stage of plaques, but downregulated in more advanced stages of atherosclerosis.[44] For the NADPH oxidase inhibitor apocynin protective effects for the vascular function are described as well.[45] Apocynin is described to inhibit Nox activity by blocking p47phox phosphorylation.[14] Thereby, apocynin has a lower effect on the Nox4/p22phox complex than on Nox2 activity.[18,46] Furthermore, apocynin has been suggested to be not a specific inhibitor of vascular Nox but rather an antioxidant.[47] Therefore, the protective effects of apocynin observed in the study by Liang *et al*. might be more due to inhibition of Nox2 rather than Nox4.[14]

The importance of Nox4-released H_2O_2 might be emphasized by the fact that loss of Nox4 led to compensation of H_2O_2 release in 26-week-old Nox4$^{-/-}$ mice. This upregulation to a level found in wild-type mice was partially blocked by NOS inhibitor L-NAME. In agreement with these findings, nNOS mRNA expression was significantly elevated in the older Nox4$^{-/-}$ mice compared with wild-type mice. A role for nNOS in vascular function has been

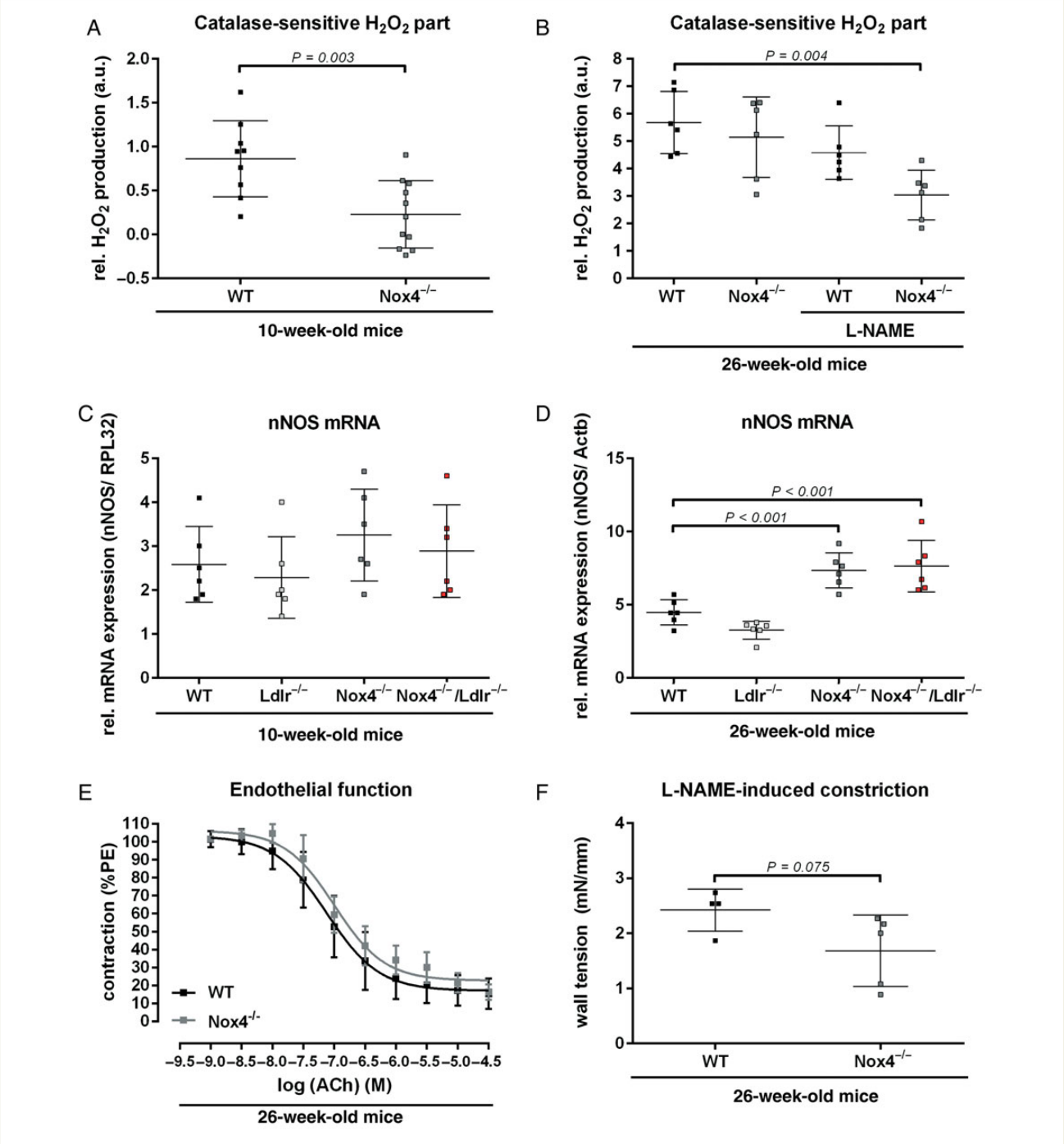


Figure 3 Compensation of hydrogen peroxide release during aging in Nox4$^{-/-}$ mice. (*A* and *B*) Amplex red assay for hydrogen peroxide generation of *Aorta thoracalis* segments of 10-week-old wild-type and Nox4$^{-/-}$ mice ($n \geq 9$) or 26-week-old mice with or without L-NAME preincubation ($n \geq 6$). H_2O_2 formation was measured as the catalase-sensitive part of the signal. (*C* and *D*) Real-time polymerase chain reaction of murine aorta from 10- and 26-week-old wild-type, Ldlr$^{-/-}$, Nox4$^{-/-}$, and Nox4$^{-/-}$/Ldlr$^{-/-}$ mice ($n \geq 6$). (*E*) Concentration–response curves of acetylcholine in aortic segments of 26-week-old wild-type and Nox4$^{-/-}$ mice ($n \geq 12$) precontracted with phenylephrine. (*F*) L-N^{G}-Nitroarginine methyl ester induced constriction of aortic segments of 26-week-old wild-type and Nox4$^{-/-}$ mice ($n \geq 4$).

described.[48] In eNOS knockdown mice, nNOS inhibition reduced H_2O_2 production and further reduced vasorelaxation.[49]

Finally, we analysed flow-mediated dilation in saphenous arteries of 26-week-old wild-type and Nox4$^{-/-}$ mice using OCT. We observed a significantly lower flow-mediated dilation in the Nox4$^{-/-}$ mice compared with wild-type mice. Although *ex vivo* analysis of vascular function in the aorta of Nox4$^{-/-}$ mice showed no difference in endothelium-dependent relaxation compared

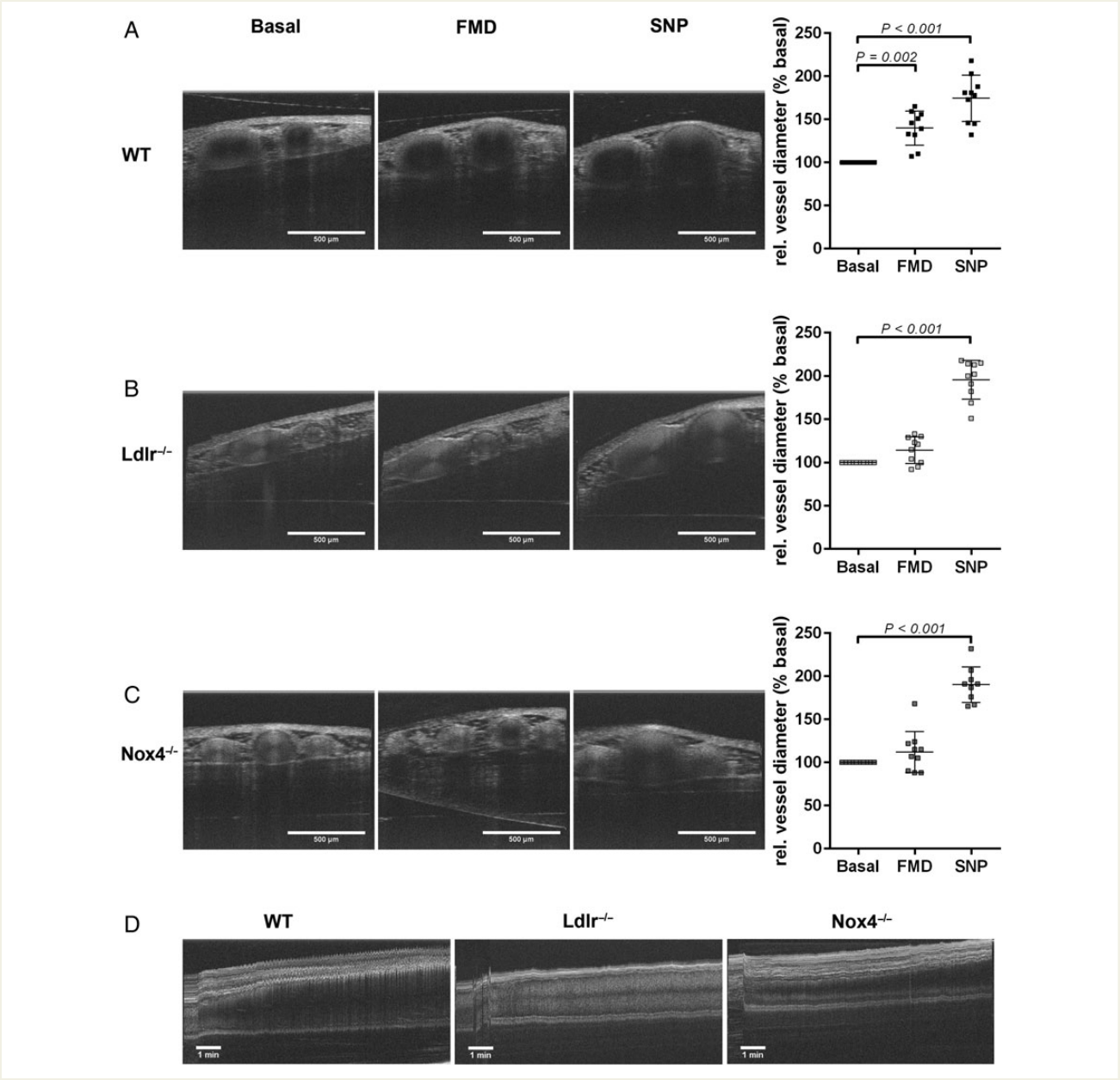


Figure 4 Nox4$^{-/-}$ mice show altered flow-mediated dilation *in vivo*. (*A*) Representative optical coherence tomography images of *Arteria saphena* and evaluation of images of 26-week-old (A) wild-type, (*B*) Ldlr$^{-/-}$, and (*C*) Nox4$^{-/-}$ mice under control conditions (basal), flow-mediated dilation and after application of sodium nitroprusside. (*D*) Representative optical coherence tomography scan of the *Arteria saphena* of 26-week-old wild-type, Ldlr$^{-/-}$, and Nox4$^{-/-}$ mice showing the process of flow-mediated dilation during 10 min after occlusion ($n = 10$).

with wild-type mice, flow-mediated dilation in saphenous artery in the Nox4$^{-/-}$ mice was significantly reduced. This supports a much higher sensitivity of the *in vivo* method to objectify alterations in vessel function. While *ex vivo* vessel ring analyses are based on the effects of pharmacological stimuli, the OCT approach allows us to monitor the effects of endogenous mechanical and chemical stimuli. Furthermore, this novel approach allows measurements of vasodynamics of a blood vessel segment integrated in the complex anatomical and physiological vascular network. Our data show evidence for a role of Nox4-released H_2O_2 in vascular function of smaller arteries like the saphenous artery and vessels of microcirculation. Paravicini *et al*. showed a role for Nox-derived H_2O_2 in flow-induced cerebral vasodilation by performing cranial window preparation in rats.[50] Catalase transgenic mice show impaired endothelium-dependent relaxation in mesenteric resistance arteries. These mice also revealed a reduction in blood flow recovery and capillary formation after hindlimb ischaemia and disorganized microvasculature.[51] Significantly attenuated blood flow recovery was also described for Nox4$^{-/-}$ mice.[33]

In conclusion, Nox4 plays an important role under physiological and pathological conditions in maintaining endothelial function. In states of increased proatherosclerotic risk such as hypercholesterolemia, Nox4 can protect against endothelial dysfunction and atherosclerosis.

Authors' contributions

H.L., C.B.: performed statistical analysis; C.B., S.R.B., A.D., E.K., H.M.: handled funding and supervision; H.L., C.B., M.B.: acquired the data; H.L., C.B., A.D., E.K., H.M.: conceived and designed the research; H.L., C.B., H.M.: drafted the manuscript; H.L., C.B., A.H., P.C., S.R.B., A.D., E.K., H.M.: made critical revision of the manuscript for key intellectual content.

Supplementary material

Supplementary material is available at *European Heart Journal* online.

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Conflict of interest: none declared.

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