Functional alterations in endothelial NO, PGI2 and EDHF pathways in aorta in ApoE/LDLR−/− mice

Gábor Csányi a,1, Mariusz Gajda b, Magdalena Franczyk-Zarow c, Renata Kostograys c, Pawel Gwożdź a, Lukasz Mateuszuk d, Magdalena Sternak a, d, Luiza Wojcik e, Teresa Zalewska e, Michal Walski e, Stefan Chlopicki a, d, e

a Department of Experimental Pharmacology, Chair of Pharmacology, Jagiellonian University Medical College, 16 Grzegórzecka Street, 31–531 Krakow, Poland
b Chair of Histology, Jagiellonian University Medical College, 7 Kopernika Street, 31–034 Krakow, Poland
c Department of Human Nutrition, Faculty of Food Technology, Agricultural University of Krakow, 122 Balica Street, 30–149 Krakow, Poland
d Jagiellonian Centre for Experimental Therapeutics (J CET), 14 Bobrzyńskiego Street, 30–348 Krakow, Poland
e Medical Research Centre, Polish Academy of Sciences, 5 Pawinskiego Street, 02–106 Warsaw, Poland

Abstract

Adequate endothelial production of nitric oxide (NO), endothelium-derived hyperpolarizing factor (EDHF), and prostaclin (PGI2) is critical to the maintenance of vascular homeostasis. However, it is not clear whether alterations in each of these vasodilatory pathways contribute to the impaired endothelial function in murine atherosclerosis. In the present study, we analyze the alterations in NO-, EDHF- and PGI2-dependent endothelial function in the thoracic aorta in relation to the development of atherosclerotic plaques in apoE/LDLR−/− mice. We found that in the aorta of 2-month-old apoE/LDLR−/− mice there was no lipid deposition, subendothelial macrophage accumulation; and matrix metalloproteinase (MMP) activity was low, consistent with the absence of atherosclerotic plaques. Interestingly, at this stage the endothelium was already activated and hypertrophic as evidenced by electron microscopy, while acetylcholine-induced NO-dependent relaxation in the thoracic aorta was impaired, with concomitant upregulation of cyclooxygenase-2 (COX-2)/PGI2 and EDHF (epoxyeicosatrienoic acids, EETs) pathways. In the aorta of 3–6-month-old apoE/LDLR−/− mice, lipid deposition, macrophage accumulation and MMP activity in the intima were gradually increased, while impairment of NO-dependent function and compensatory upregulation of COX-2/PGI2 and EDHF pathways were more accentuated.

These results suggest that impairment of NO-dependent relaxation precedes the development of atherosclerosis in the aorta and early upregulation of COX-2/PGI2 and EDHF pathways may compensate for the loss of the biological activity of NO.

© 2012 Elsevier Inc. All rights reserved.

1. Introduction

There is overwhelming evidence demonstrating that proper function of endothelium guards the health of the cardiovascular system, while endothelial dysfunction is associated with cardiovascular diseases, including atherosclerosis, diabetes mellitus, and hypertension [1,2]. Endothelial dysfunction is a systemic pathological state of the endothelium and is broadly defined as an impairment of vascular relaxation, due to decreased nitric oxide (NO) production by the endothelium and/or increased inactivation of NO. In particular, it was demonstrated that impairment of NO-mediated vasodilatation precedes the development of atherosclerosis in humans, and that assessment of NO-dependent vasodilatation has a prognostic and therapeutic significance in atherosclerosis [3,4]. Similarly, in diet-induced animal models of atherosclerosis [5–7] and gene-targeted mouse models of atherogenesis, impaired NO-mediated relaxation was repeatedly demonstrated [8]. On the other hand, there are some studies that reported normal endothelium-dependent relaxation in murine atherosclerosis [9–11].

The exact mechanisms that lead to impairment of endothelium-dependent relaxation during atherosclerosis are still not known. Reduced nitric oxide synthase (NOS) activity, decreased availability of substrate and co-factor (l-arginine and tetrahydrobiopterin) for NO synthesis, enhanced inactivation of NO (increased oxidative stress), and decreased smooth muscle...
cell sensitivity to NO have been shown to contribute to impaired NO-dependent vasorelaxation [12–15]. At the same time, many reports have debated these mechanisms [16,17].

Although NO is recognized as the primary vasodilator in conduit vessels, there is evidence for the role of other endothelium-derived vasodilators, especially in small arteries [18,19]. Indeed, it is well known that endothelial cells produce not only NO, but other vasodilators, such as prostacyclin (PGI2) and endothelium-derived hyperpolarizing factor (EDHF) [2]. Since the contribution of PGI2 and EDHF is different in conduit, medium-sized and small arteries, the changes in their action are of critical importance for the local regulation of blood flow, peripheral vascular resistance, blood pressure, platelet adhesion to the endothelium, and vascular inflammation, all of which are known to play a role in atherogenesis. Quite surprisingly, though most previous studies have investigated the relative contribution of NO to endothelium-dependent relaxation in atherosclerosis, there are only a few controversial reports on the role of PGI2 and EDHF in endothelial function during atherogenesis. A previous study reported that diminished EDHF activity may be a factor contributing to decreased endothelium-dependent relaxation in patients with hypercholesterolemia [20], while other studies demonstrated that increased activity of EDHF may compensate for endothelial abnormalities in cardiovascular disorders [21,22]. Moreover, contradictory results were reported regarding the role of PGI2 in endothelial dysfunction in atherosclerosis [23–25].

The discrepancies between results obtained previously may be due to the use of different experimental models, assessment of endothelial function in different types of vessels at different time points during the development of atherosclerosis, and investigating the contribution of only one vasodilator to vascular relaxation and disregarding the role of other vasodilators in vascular function.

To our knowledge there are still no reports that have simultaneously analyzed the functional alterations in endothelial production of NO, PGI2 and EDHF in athero-prone conduit vessels along the development of atherosclerosis. Accordingly, the aim of the present study was to assess possible changes in the production of PGI2 and EDHF that are associated with the development of the impairment of NO-dependent function in the thoracic aorta of apoE/LDLR−/− mice along atherosclerosis development.

2. Materials and methods

The animal procedures conform with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85–23, revised 1996) and the experimental procedures used in the present study were approved by the local Jagiellonian University Ethical Committee on Animal Experiments.

2.1. Animals

ApoE/LDLR−/− mice on C57BL/6J background and wild-type C57BL/6J mice from The Jackson Laboratory (Maine, USA) were bred in the animal house of Agricultural University in Krakow, Poland. The mice were maintained on 12-h dark/12-h light cycles in air-conditioned rooms with access to standard rodent chow diet and water ad libitum. At the age of 2, 3, 4, 6 and 8 months, apoE/LDLR−/− and age-matched C57BL/6J mice were injected intraperitoneally with 1000 IU of fraxiparine (Sanofi-Synthelabo, Santea, France) and sacrificed under anesthesia (thiopental, 100 mg/kg i.p.).

2.2. Measurement of plasma lipoproteins

Blood was collected from the right ventricle. Plasma was separated by centrifugation at 1000 x g at 4°C for 10 min and was immediately frozen and stored at −80°C. The lipid profile was analyzed using commercially available kits for HDL-, LDL- (Olympus Diagnostica GmbH, Hamburg, Germany), total-cholesterol and triglycerides (CORMAY, Lublin, Poland).

2.3. Vascular inflammation and quantification of atherosclerosis

2.3.1. Lipid deposition in the whole aorta and in the aortic roots

The method of the quantification of the atherosclerotic plaques in the whole aorta (“en face”) and in the aortic root (“cross section”) was described previously [26]. Briefly, after opening the thorax, the heart and the aorta were perfused by phosphate buffered saline (PBS, pH 7.4). After cleaning, the whole aorta from the arch to the bifurcation was dissected from the surrounding tissues and fixed in 4% formaldehyde. Then it was opened longitudinally, pinned onto brown silicon plates and stained with Sudan IV (Sigma–Aldrich, St. Louis, MO, USA). The aortic lesion and the total aortic area were calculated automatically using Aphelion software, and originally designed algorithms as described previously [27].

The heart and the ascending aorta were removed and embedded in optimal cutting temperature (OCT) compound (CellPath, UK) and snap-frozen. Ten micrometer-thick cryosections were cut from the aortic root using a Leica Jung Cryocut CM1800 microtome with a standardized protocol. After fixation (4% paraformaldehyde; pH 7.0) the sections were stained with Meyer’s hematoxylin and oil red-O (ORO) (Sigma–Aldrich, USA). The total area of the lesion was measured semi-automatically in each slide using LSM Image Browser 3 software (Zeiss, Jena, Germany). For each animal a mean lesion area was calculated from eight sections.

2.3.2. Quantification of inflammation in atherosclerotic plaques by CD68 staining

Acetone-fixed sections from the aortic root were incubated overnight with primary antisera. For detection of CD68, rat anti-mouse CD68 (Serotec, Oxford, UK) and goat anti-rat IgG biotinylated antibodies, followed by DTAF-conjugated streptavidin (both from Jackson IR, West Grove, PA, USA) were used. Sections were examined using an epifluorescence Olympus BX50 microscope equipped with appropriate filter cubes to show Cy3 (red) and DTAF (green) fluorescence. Images were registered with a Camea C5050 digital camera. In each section, the total area occupied by CD68-immunopositive macrophages was measured semi-automatically using LSM Image Browser software and was expressed in absolute values (mm²) as well as a percentage of cross-section area stained by oil red-O (CD68/ORO). For each animal, the CD68 positive area was calculated from eight sections.

2.3.3. Quantification of inflammation in atherosclerotic plaques by in situ zymography

Gelatinolytic activity of matrix metalloproteinases (MMP-2 and MMP-9) in atherosclerotic plaque sections was analyzed by in situ zymography [28]. Frozen, non-fixed, 10 µm-thick cryosections of aortic roots were thawed and incubated for 2 h at 37°C in a humid dark chamber in 100 µl of reaction buffer containing 50 mg/ml of FITC-labeled DQ-gelatin (Molecular Probes, Eugene, OR) that was intramolecularly quenched. Gelatin-FITC cleavage by tissue gelatinases released peptides whose fluorescence was representative of net proteolytic activity of tissue MMPs. Sections were rinsed in PBS and fixed in cold 4% paraformaldehyde for 15 min, then mounted in mounting medium (Dako) and observed using fluorescence microscopy.

The fluorescence intensity of atherosclerotic plaques was measured in sections at the same level above aortic valves. In control animals without atherosclerotic plaques, fluorescence was measured approximately at the same level over the intima of
2.4. Analysis of endothelial function in isolated aorta

The middle part of the descending thoracic aorta was quickly removed and after cleaning it was cut into 3 rings each approximately 3-mm in length. Vascular rings were then transferred to organ-bath chambers filled with 5 ml of Krebs-Heinselait solution (mM: NaCl 118, KCl 4.7, CaCl2 2.5, MgSO4 1.2, NaHCO3 25, KH2PO4 1.2, glucose 10, pyruvic acid 2, HEPES 10 and EDTA 0.03) maintained at 37 °C. pH 7.4 and gassed with 95% O2 and 5% CO2. The rings were mounted between 2 hooks, attached to an isometric force transducer (Hugo Sachs Electronic-Harvard Apparatus, Germany) that was connected to a recorder (Graphtec WR3320, UK) for continuous recording of tension. After an equilibration period of 30 min, resting tension was stepwise increased to reach the final value of 0.75 g. The rings were then incubated further for 30 min. Stretching of the aortic rings to a resting tension of 0.75 g was chosen on the basis of our preliminary experiments in which it was found to result in optimal length–tension relationship.

The viability of the vessels was documented by a contractile response to potassium chloride (KCl, 30 mM). Aortic rings were then precontracted with phenylephrine to reach approximately 80% of KCl-induced contraction (Phe, 10−6 to 3 × 10−6 M). After reaching a stable plateau phase, a cumulative concentration-dependent response for acetylcholine (Ach, 10−8 to 10−6 M) was induced. The endothelium-independent vasorelaxation was evoked by S-Nitroso-N-acetylpenicillamine (SNAP, 10−9 to 10−6 M). Basal NOS production was measured on mildly precontracted rings (10−20% of KCl-induced contraction) based on the contraction induced upon the administration of the nitric oxide synthase (NOS) inhibitor Nω-nitro-L-arginine methyl ester (L-NAME, 300 μM, 15 min).

In some experiments, the cyclooxygenase (COX) inhibitor indomethacin (5 μM, 30 min) was used to test the role of COX metabolites in Ach-induced vasodilation. EDHF-mediated relaxation was determined as the magnitude of Ach-induced relaxation in the presence of L-NAME and indomethacin. The identity of EDHF was analyzed by pretreatment with the Ca2+-activated K+ channel blocker tetraethylammonium chloride (TEA, 10 mM) or the epoxysynapine synthesis inhibitor miconazole (MICO, 10 μM). For vascular function analysis in each experimental group, at least n = 6 animals with 3 rings from each animal were used.

2.5. Determination of COX-expression and basal prostacyclin production in the aorta

Aortic homogenates were subjected to SDS-PAGE. Blots were probed with COX-1 (Cell Signaling Technology Inc., Danvers, MA) and COX-2 (Abcam, San Francisco, CA) antibodies and incubated with their respective secondary antibodies (IRDye antibodies; LI-COR Biotechnology, Lincoln, NE, USA). Blots were scanned using the Odyssey Infrared Imaging System. Densitometry was quantified using Image J (NIH).

Basal PGI2 production by the aortic rings was analyzed by measuring its stable hydrolysis product 6-keto-PGF1α in organ bath medium. Commercially available EIA kits from R&D Systems (Minneapolis, MN, USA) were used. The aortic rings were preincubated for 15 min in fresh Krebs-Henseleit solution maintained at 37 °C, pH 7.4 and gassed with 21% O2 and 5% CO2. After washing, the rings were incubated again for 30 min in Krebs-Henseleit solution. Samples of supernatant were then collected and processed for level of 6-keto-PGF1α according to the manufacturer’s instructions. The enzymatic source of PGI2 was assessed using the non-selective COX inhibitor indomethacin (5 μM) or the selective COX-2 inhibitor rofecoxib (25 μM). Aortic rings were collected after the experiments, dried for 48 h at 60 °C, and weighed. Prostacyclin production was expressed as pg/mg of dry weight of aortic rings.

2.6. Measurement of hydrogen peroxide (H2O2) production in the aorta

H2O2 levels were measured in the aorta of ApoE/LDLR−/− and C57BL/6j mice by Amplex Red. Briefly, thoracic aortas were homogenized in ice-cold lysis buffer (PBS containing protease inhibitor cocktail, and 0.1 mM PMSF). Measurement of H2O2 levels was performed in assay buffer (25 mM Hepes, pH 7.4, containing 120 mM NaCl, 3 mM KCl, 1 mM MgCl2, 0.1 mM Amplex Red, and 0.32 U/ml of HRP) for 30 min at room temperature. Fluorescence measurements were made using a Biotek Synergy 4 Hybrid Multi-Mode Microplate Reader (Fluorescence excitation: 530 and Fluorescence emission: 590). Conformation that H2O2 was the oxidant responsible for the fluorescence signal was accomplished by inhibition of fluorescence by exposure to catalase (3000 U/ml).

2.7. Statistical analysis

The results are expressed as the mean ± SEM. Comparison of the means was assessed by ANOVA and post hoc Scheffe’s test. p < 0.05 was considered to be statistically significant.

3. Results

3.1. Development of hypercholesterolemia in apoE/LDLR−/− mice

As shown in Table 1, total, LDL, and HDL cholesterol levels were elevated in 2-month-old apoE/LDLR−/− mice and remained approximately at the same level in older animals. Triglyceride level remained unchanged in apoE/LDLR−/− mice, irrespective of the age of the animals. For comparison, typical lipid profile of wild type C57BL/6j mice is also shown (Table 1).

3.2. Development of lipid deposition in the aorta in apoE/LDLR−/− mice

Whether measured in the whole aorta (“en face”) by Sudan IV staining or in the aortic root (“cross section”) by oil red-O staining, lipid deposition was absent in the thoracic aorta from 2-month-old
apoE/LDLR−/− mice (Fig. 1A and B). In the aortic root and the distal segments of the thoracic aorta, lipid depots were visible in 4-month-old and older apoE/LDLR−/− mice. The surface covered by atherosclerotic lesions increased gradually with age in both the aortic root/arch regions and the distal segments of the thoracic aorta (Fig. 1A and 1B).

3.3. Development of vascular inflammation in apoE/LDLR−/− mice

In aortic roots from 2-month-old apoE/LDLR−/− mice, CD68-positive macrophages were not detected in the subendothelial space, but their accumulation was increasingly apparent in the aortic roots from 3- to 8-month-old apoE/LDLR−/− mice (Fig. 1C). Interestingly, the percent area of the oil red-O-stained plaque area that was covered by CD68-positive staining (CD68/ORO) was significantly higher at the early vs. late stage of atherosclerosis development (61.92 ± 0.1%, 40.23 ± 0.1% and 28.43 ± 0.03% in 3-, 4- and 8-month-old apoE/LDLR−/− mice, respectively, p < 0.05 between 3- and 8-month-old apoE/LDLR−/− mice). Similarly to CD68-positive staining, MMP activity in aortic roots of 2-month-old apoE/LDLR−/− mice was comparable to the level detected in wild-type animals. As shown in Fig. 1D, MMP activity was significantly increased in the aorta of 3-month-old apoE/LDLR−/− mice and was gradually increased in older animals.

3.4. Changes in endothelial ultrastructure

Alterations in endothelial ultrastructure were clearly visible in the aorta in as early as 2-month-old apoE/LDLR−/− mice, at which stage there was no lipid deposition in the intima of these vessels. As shown in Fig. 2, in aorta from 2-month-old apoE/LDLR−/− mice, the endothelium displayed signs of hypertrophy and activation, consisting of active hypertrophic nucleus, increased number of mitochondria and Weibel-Palade bodies, as well as activated rough endoplasmic reticulum. Monocytes adhering to the surface of the endothelium, as well as widened and remodeled elastic lamina were also visible. In the aorta from older apoE/LDLR−/− mice, typical foam cells and other typical features of atherogenesis were detected, including adhering monocytes and platelets to endothelial (Fig. 2).

3.5. Vascular function of the aorta in apoE/LDLR−/− mice

3.5.1. NO-dependent vascular function

In the aorta from control C57BL/6J mice, endothelium-dependent relaxation induced by Ach was similar, irrespective of the age of the animals (101.03 ± 6.09%, 96.13 ± 2.36% and 108.87 ± 3.97% for Ach 10−6 M in 2-, 4- and 6-month-old C57BL/6J mice, respectively). Endothelium-independent relaxation induced by SNAP was also similar in C57BL/6J mice, and maximal relaxation amounted to approximately 100% (103.96 ± 3.99% for SNAP 10−6 M in 6-month-old C57BL/6J mice). In contrast, Ach-induced endothelium-dependent relaxation was significantly impaired in the thoracic aorta from 2-month-old apoE/LDLR−/− mice (Ach 10−6 M: 82.37 ± 3.2%) and remained diminished in older animals (89.2 ± 3.6%, 77.83 ± 3.3% and 72.2 ± 3.1% for Ach 10−6 M in 4-, 6- and 8-month-old apoE/LDLR−/− mice, respectively, p < 0.05 in apoE/LDLR−/− vs. C57BL/6J mice) (Fig. 3). The SNAP-induced endothelium-independent relaxation in aorta was nearly identical for 2-, 4- and 6-month-old apoE/LDLR−/− mice (90.55 ± 3.66%, 97.32 ± 2.94% and 105.8 ± 2.77% for SNAP 10−6 M in 2-, 4- and 6-month-old apoE/LDLR−/− mice, respectively). Interestingly, SNAP response was significantly impaired in 8-month-old animals (82.03 ± 2.79% for SNAP 10−6 M, p < 0.05 in apoE/LDLR−/− vs. C57BL/6J mice).

Basal NO production, determined as the magnitude of L-NAME–induced vasoconstriction in phenylephrine- preconstricted vessels, was diminished in 2- and 4-month-old apoE/LDLR−/− mice (16.66 ± 5.3% and 17.63 ± 8.62%, respectively) as compared to age-matched wild-type animals (31.98 ± 3.05 and 33.32 ± 8.17%, respectively, p < 0.05). Interestingly, in 6- and 8-month-old apoE/LDLR−/− mice, the magnitude of L-NAME induced response was restored (33.79 ± 4.13% and 37.8 ± 4.12%, respectively).

3.5.2. COX-dependent vascular function

Preincubation of aortic rings with indomethacin did not modify the magnitude of Ach-induced relaxation, either in control or in apoE/LDLR−/− mice (data not shown). Basal PGF2α production of aorta in C57BL/6J mice (as assessed by the level of 6-keto-PGF1α in supernatant of aortic rings) was slightly increased with age (in pg/mg: 145.8 ± 52.4, 160.9 ± 71.3 and 202.7 ± 46.3 for 2-, 4- and 6-month-old C57BL/6J mice, respectively). In the presence of indomethacin, PGF2 production in the aorta from C57BL/6J mice was entirely inhibited, while rofecoxib had no effect (Fig. 4). In contrast, apoE/LDLR−/− mice developed a dramatic age-dependent upregulation of PGF2 production in aorta. Prostacyclin production of aorta in apoE/LDLR−/− mice was entirely inhibited by indomethacin and partially inhibited by rofecoxib (Fig. 4).

COX-1 expression did not change significantly in the aorta of ApoE/LDLR−/− mice (COX-1/β-Actin ratio were: 0.66 ± 0.01, 0.61 ± 0.01, 0.73 ± 0.03 and 0.73 ± 0.02 for 2-month-old C57BL/6J, and 2-, 4- and 6-month-old ApoE/LDLR−/− mice, respectively). In contrast, COX-2 expression slightly decreased with age in ApoE/LDLR−/− mice (COX-2/β-Actin ratio were: 0.30 ± 0.02, 0.25 ± 0.01, 0.18 ± 0.01* and 0.20 ± 0.01* for 2-month-old C57BL/6J, and 2-, 4- and 6-month-old ApoE/LDLR−/− mice, respectively, ‘p < 0.05).

3.5.3. EDHF-dependent vascular function

In aortic rings from C57BL/6J mice, the combined preincubation with L-NAME (300 μM) and indomethacin (5 μM)
Fig. 1. (A and B). Representative images and summarized data showing Sudan IV-stained lesions in the whole aorta (A) [magnification 3×] and oil red-O-stained lesions in the aortic roots (B) [magnification 80×] in 2- to 8-month-old apoE/LDLR<sup>−/−</sup> mice. Data represent the means ± SEM of n = 4–5 animals in each group; * indicates p < 0.05 and *** indicates p < 0.001 vs. 2-month-old apoE/LDLR<sup>−/−</sup> mice. (C) Representative images and summarized data of CD68 immunostaining showing the accumulation of macrophages in the subendothelial space of aortic roots in 2- to 8-month-old apoE/LDLR<sup>−/−</sup> mice. Data represent the means ± SEM of n = 4–7 animals in each group; * indicates p < 0.05 vs. 2-month-old apoE/LDLR<sup>−/−</sup> mice. (D) Representative images and summarized data showing MMP activity in aortic roots in 2- to 6-month-old apoE/LDLR<sup>−/−</sup> mice. Data represent the means ± SEM of at least n = 4 animals in each group; * indicates p < 0.05 vs. C57Bl/6J mice, while ∆ indicates p < 0.05 vs. 2-month-old apoE/LDLR<sup>−/−</sup> mice.
almost completely blocked Ach (10−6 M)-induced vasodilation (5.79 ± 3.71%, 6.57 ± 4.18% and 4.74 ± 2.45% for 2-, 4- and 6-month-old C57BL/6J mice, respectively) (Fig. 5A).

In contrast, in apoE/LDLR−/− mice the EDHF-dependent vasodilation was gradually up-regulated starting from 2-month-old mice. The magnitude of Ach (10−6 M)-induced response in the presence of l-NAME and indomethacin amounted to 16.87 ± 4.55%, 40.61 ± 11.84%, 33.03 ± 4.74% and 32.92 ± 2.72% for 2-, 4-, 6- and 8-month-old apoE/LDLR−/− mice, respectively (p < 0.05, apoE/LDLR−/− vs. C57BL/6J mice) (Fig. 5A).

The EDHF-component of Ach-induced vasodilation was abrogated in apoE/LDLR−/− mice if KCl (30 mM) was used to preconstrict the vessels instead of phenylephrine (<3% both in apoE/LDLR−/− and C57BL/6J mice, respectively). In addition, the EDHF-dependent vasodilation in the thoracic aorta was completely blocked by preincubation with tetraethylammonium chloride (TEA, 10 mM) – an antagonist of Ca2+–activated K+ channels, or with miconazole (MICO, 10 μM) – a selective inhibitor of epoxyeicosatrienoic acid (EET) synthesis (Fig. 5B).

3.6. H2O2 production in the aorta of apoE/LDLR−/− mice

H2O2 production in the aorta of 2- and 4-month-old apoE/LDLR−/− mice was comparable to that of wild type controls (RFU values were: 1204 ± 6.9, 1319 ± 36.7 and 1150 ± 79.69 for 2-month-old C57BL/6J, and 2- and 4-month-old apoE/LDLR−/− mice, respectively). In contrast, H2O2 production in the aorta of 6-month-old apoE/LDLR−/− mice was significantly increased as compared to wild type control (RFU values were: 1204 ± 6.9 and 1412 ± 37.6 for C57BL/6J and 6-month-old apoE/LDLR−/− mice, respectively, p < 0.05).

![Graph](image1.png)

**Fig. 3.** Impairment of endothelium-dependent vasodilation in isolated aortic rings from 2- to 8-month-old apoE/LDLR−/− mice as compared to 6-month-old C57BL/6J mice. Data represent the means ± SEM of n = 6–9 animals in each group; * indicates p < 0.05 vs. C57BL/6J mice; *** indicates p < 0.001 vs. C57BL/6J mice.

![Graph](image2.png)

**Fig. 4.** Upregulation of basal PGI2 production (measured as 6-keto-PGF1α) by the aortic rings from apoE/LDLR−/− mice at various age as compared to C57BL/6J mice (6 months of age). The enzymatic source of PGI2 was assessed using the non-selective COX inhibitor indomethacin (5 μM) or the selective COX-2 inhibitor rofecoxib (25 μM). Prostaglandin production was expressed as pg/mg of dry weight of aortic rings. Data represent the means ± SEM of n = 5 animals in each group. * indicates p < 0.05 vs. C57BL/6J mice.
4. Discussion

In the present work, in apoE/LDLR−/− mice fed chow diet, we comprehensively analyzed the functional alterations in NO-, PG12- and EDHF-dependent endothelial pathways in aorta in relation to the development of lipid deposits and vascular inflammation characteristic for atherosclerotic plaque development. Our results demonstrated that impairment of NO-dependent relaxation in conduit vessels of apoE/LDLR−/− mice occurs prior to the development of atherosclerosis, supporting the key role of dysfunctional endothelium in the initiation of atherogenesis in this mouse model of atherosclerosis. Moreover, we observed for the first time that COX-2-derived PG12 production and EDHF-mediated relaxation were increased in the aorta of apoE/LDLR−/− mice. These findings suggest that impairment of NO-dependent relaxation precedes the development of atherosclerosis in the aorta, and upregulation of COX-2/PG12 and EDHF pathways that occurs early along the development of atherosclerosis may compensate for the loss of the biological activity of NO.

Total- and LDL-cholesterol levels were highly elevated in apoE/LDLR−/− mice from the early period of life and over the time of several months resulted in marked atherosclerotic lesion formation in the aortic root, the aortic arch, and in the distal segments of the descending thoracic aorta [30]. At the age of 2-months, however there was no evident lipid deposition (as assessed by Sudan IV and oil red-O), no subendothelial macrophage accumulation (CD68 staining), and no increased MMP activity (gelatinase activity assessed by in situ zymography). However, at this stage acetylcholine-induced NO-dependent relaxation in the thoracic aorta was already impaired, and there was a decrease in basal NO production as assessed by the magnitude of l-NAME-induced response.

Interestingly, the early impairment of endothelial NO-dependent function was associated with the compensatory upregulation of COX-2/PG12 and EDHF pathways. Here, we demonstrated that basal PG12 production in aorta from C57BL/6j mice was slightly increased with age and was entirely COX-1-mediated. In contrast, PG12 production in the aorta from 2-month-old apoE/LDLR−/− mice was already increased and it was entirely COX-2-mediated. In the aorta from 4- and 6-month-old apoE/LDLR−/− mice PG12 production was further increased and it was mostly COX-2-mediated with only partial contribution from COX-1. Interestingly, expression of COX-1 did not change in apoE/LDLR−/− mice, while expression of COX-2 modestly declined in the aorta from 4- and 6-month-old ApoE/LDLR−/− mice, suggesting that alterations in PG12 production observed in the present work are mostly if not uniquely functional in nature.

Increased vascular PG12 production was also shown in apoE−/− mice fed Western diet [31] and in patients with atherosclerosis [32]. Elegant work by Kobayashi et al. showed that in apoE−/− mice lacking PG12 receptor, atherogenesis was markedly accelerated [33]. COX-2-mediated compensatory increase in basal PG12 production demonstrated in the present work may thus play an important defensive role compatible with the notion of vasculoprotective and anti-atherogenic activity of COX-2/PG12 pathway [34].

Along with the upregulation of COX-2/PG12 pathway, there was also an upregulation of the NOS- and COX-independent component of Ach−induced vasodilation that could be attributed to EDHF. Indeed, this component of Ach−induced vasodilation was not affected by indomethacin, but was abolished by elevated extracellular K+ concentration (30 mM), pretreatment with Ca2+-activated K+ channel blocker tetraethylammonium chloride (TEA, 10 mM) (grey bar) or l-NAME, indomethacin and the COX-2/PG12 inhibitor miconazole (MICo, 10 μM) (black bar). Data represent the means ± SEM of n = 6–9 animals in each group. ** indicates p < 0.01 and *** indicates p < 0.001 vs. l-NAME + indomethacin alone.
reticulum calcium ATPase (SERCA) in the vascular wall [7] that may contribute to the impairment of vasoprotective cGMP-dependent function at the late stage of atherosclerosis [42].

In the present work we did not investigate the mechanism of the upregulation of COX-2/PGL2 and EDH pathways that occurred along with the impairment of NO-dependent function in atherosclerosis. We however measured H2O2 production in the aorta of apoE/LDLR−/− mice and found that H2O2 production was not increased in the aorta of 2- and 4-month-old apoE/LDLR−/− mice as compared to wild type controls but it was increased in the aorta of 6-month-old apoE/LDLR−/− mice. Previous studies demonstrated that hypercholesterolemia in mice results in activation of NADPH oxidase [43] and xanthine oxidase [44], leading to increased superoxide production [45] and decreased NO-dependent function that could depend on NO-scavenging, BH4 deficiency and NOS uncoupling [43]. Indeed, overexpression of GTP-cyclodrolase I improved endothelial function and reduced atherosclerosis in apoE−/− mice [46]. Our data suggest that oxidative stress, which accompanied endothelial dysfunction at the later stages of atherosclerosis development, may contribute to increased activity of COX-2/PGL2 pathway. However, H2O2 does not seem to operate as an EDHF in this model. More comprehensive approach to analyze the contribution of reactive oxygen species to mechanisms of changes in COX-2/PGL2 and EETs-dependent pathways along atherosclerosis development in apoE/LDLR−/− mice are warranted.

It is important to note that we revealed that the impairment of NO-dependent endothelial dysfunction was associated with ultrastructural signs of endothelial activation and hypertrophy. As early as in 2-month-old apoE/LDLR−/− mice, endothelial cells of conduit vessels were highly activated and contained high content of rough endoplasmic reticulum, abundant Weibel-Palade bodies, and increased number of mitochondria. These changes are suggestive of an increased level of protein biosynthesis, consistent with endothelial cell hypertrophy and highly activated endothelium featured by the impairment of vasoprotective NO activity, increased oxidative stress, as well as pro-inflammatory and pro-thrombotic phenotype that drives vascular inflammation [47,48]. In fact, our electron microscopy studies revealed monocytes adhering to the surface of the endothelial cells and activated platelets in the vicinity of the dysfunctional endothelium already in 2-month-old apoE/LDLR−/− mice. Furthermore, our result showing CD68 immunopositive macrophages and foam cells accumulating in subendothelial space of the aortic root starting from 3-month-old apoE/LDLR−/− mice, as well as gradually increasing activity of gelatinases (MMP2, MMP9), stay in line with the current understanding of the role of macrophages and macrophage-derived MMPs in the initiation of high cholesterol-driven vascular inflammation and atherosclerosis [49,50]. Indeed, metalloproteinases may contribute to the plaque progression and vascular inflammation by a number of ways, including degradation of extracellular matrix and thrombus formation [51].

It is increasingly accepted that, in humans, the development of endothelial dysfunction in conduit and resistance vessels represents an early systemic event in atherogenesis [52], and the measurement of endothelial function in patients with cardiovascular risk possesses diagnostic, prognostic, as well as therapeutic significance [1,47]. Indeed, in hypercholesterolemic patients, endothelial dysfunction in vivo was present in conduit and resistance arteries before the appearance of atherosclerotic plaques. Importantly, endothelial dysfunction in both types of arteries independently relate to cardiovascular risk, suggesting the impairment of different endothelial mechanisms [50]. In contrast, it was claimed that in apoE−/− mice with atherosclerosis, endothelial dysfunction occurs only at the late stage of the development of atherosclerotic plaques [10,53]. Interestingly, in apoE−/− and LDLR−/− mice, vasoactive endothelial function of isolated skeletal muscle arteries was preserved, and impairment of NO activity was compensated by 12/15 lipooxygenase products [54]. Also in cremaster arteries in vivo, endothelial function was largely preserved despite long-standing hypercholesterolemia in mice fed a cholesterol-enriched diet [55]. Importantly, our data, showing impairment of NO-dependent relaxation prior to the development of atherosclerosis in apoE/LDLR−/− mice, suggest that atherosclerosis in this model could resemble, to some extent, the human disease. Some of the previous discrepancies in the literature may relate to the differences in endothelial response to hypercholesterolemia and existing compensatory mechanisms in different vessel types. However, in regard to conduit vessels, we confirmed that endothelial inflammatory response constitutes the early sign of cholesterol-driven atherogenesis in apoE/LDLR−/− mice. In the aorta from apoE/LDLR−/− mice, the endothelial response evident as endothelial activation and hypertrophy on the level of electron microscopy, encompasses the impairment of endothelial NO-dependent biological activity and concomitant upregulation of PGL2 and EETs pathways. The ability of aortic endothelium to compensate for the loss of NO with increased synthesis of EETs and PGL2 may play an important compensatory role in atherosclerosis. Consequently, pharmacology of endothelium aimed to boost endogenous EETs [56] or COX-2 derived PGL2 pathway [57] may prove effective in atherosclerosis.

Acknowledgments

This work was supported by the European Union from the resources of the European Regional Development Fund under the Innovative Economy Programme (grant coordinated by JCT-JU, No. POIG.01.01.02-00-069/09). Supplementary funding was provided by the statutory grants from Jagiellonian University Medical College.

References


AG. vascular activity of endothelium-derived relaxing factor in atherosclerotic

[15] Jorge PA, Osaki MR, de Almeida E, Credidio Neto L, Metze K. Effects of vita-
min E on endothelium-dependent coronary flow in hypercholesterolemic dogs.
Atherosclerosis 1996;126:43–51.

[16] Hamilton CA, Howie CA, Jardine E, Reid JL. Endothelium dependent and
independent relaxation of aortic rings from Watanabe heritable hyperlipi-
demic rabbits after exposure to free radical generating system. Free Radic Res

l-arginine on vascular structure and reactivity in hypercholesterolemic rabbits.

[18] Knockert AD, Agee TJ, Kubicki CE. Endothelium-derived hyperpolarizing
factor in vascular physiology and cardiovascular disease. Atherosclerosis

[19] Shimokawa H, Yatsutake H, Fujii K, et al. The importance of the hyperpo-
larizing mechanism increases as the vessel size decreases in endothelium-
dependent relaxations in rat mesenteric circulation. J Cardiovasc Pharmacol

Importance of endothelium-derived hyperpolarizing factor in human arteries.

pathways in the aortic endothelium after myocardial infarction in rats. Eur J
Heart Fail 2006;8:769–76.


[23] Vaphiades PM. Endothelial dysfunction: the first step toward coronary arte-

[24] Smith DD, Tan X, Tawfik OL, Milne G, Stechschulte DJ, Dileepan KN. Increased aor-
tic atherosclerotic plaque development in female apolipoprotein E-null mice is
associated with elevated thromboxane A2 and decreased prostacyclin pro-


ion of atherosclerosis in apolipoprotein E and low density lipoprotein receptor

naturally enriched with conjugated linoleic acid, on the blood lipid profile,
development of atherosclerosis and composition of atherosclerotic plaque in
apolipoprotein E and low-density lipoprotein receptor double-knockout mice

[28] Rivera S, Ogier C, Jourquin J, et al. Gelatinase B and TIMP-1 are regulated in a
cell- and time-dependent manner in association with neuronal death and glial

[29] Chlipiocki S, Walski M, Bartus JB. Ultrastructure of immediate microvascular
lung injury induced by bacterial endotoxin in the isolated, non-deficient lung

of lipoprotein clearance: tests of the hypothesis in knockout mice lacking the
low density lipoprotein receptor, apolipoprotein E, or both proteins. Proc Natl

[31] Belton OA, Duffy A, Toomey S, Fitzgerald DJ. Cyclooxygenase isoforms and
platelet vessel wall interactions in the apolipoprotein E knockout mouse model

[32] Fitzgerald GA, Smith B, Pedersen AK, Brash AR. Increased prostacyclin biosyn-

[33] Kobayashi T, Tahara Y, Matsumoto M, et al. Roles of thromboxane A2(2) and
prostacyclin in the development of atherosclerosis in apoE-deficient mice. J

[34] Grosser T, Friets S, Fitzgerald GA. Biological basis for the cardiovascular conse-


[36] Pfister SL, Falck JR, Campbell WB. Enhanced synthesis of epox-
yeicosatrienoic acids by cholesterol-fed rabbit aorta. Am J Physiol 1991;261:
H843–52.

[37] Metzner M, Schildknecht S, Ullrich V. Redox regulation of vascular
prostanoid synthesis by the nitric oxide-superoxide system. Biochem Biophys

[38] Bauer J, Popp R, Hecker M, Sauer E, Fleming I, Busse R. Nitric oxide attenu-
ates the release of endothelium-derived hyperpolarizing factor. Circulation

epoxide-isomerase-derived eicosanoid HMG-CoA reductase inhibitors in the
forefront of pharmacology of endothelium. Pharmacol Rep 2005;57 Suppl 4:
86–96.

[40] Walski M, Chlipiocki S, Celary-Walska R, Frontczak-Baniewicz M. Ultrastruc-
tural alterations of endothelium covering advanced atherosclerotic plaque in
human carotid artery visualised by scanning electron microscope. J Physiol

[41] Harsission GK, Robertson AK, Soderberg-Naucler C. Inflammation and atheroscle-

[42] Newby AC. Metalloproteinases and vulnerable atherosclerotic plaques. Trends

[43] Libby P. The molecular mechanisms of the thrombotic complications of

[44] Anderson TJ, Gerhard MD, Meredith IT, et al. Systemic nature of endothelial

dysfunction in apolipoprotein E knockout mice. Arterioscler Thromb Vasc Biol

[46] Stapleton PA, Goodwill AG, James ME, Frisbee JC. Altered mechanisms of
endothelium-dependent dilation in skeletal muscle arterioles with genetic hyp-

[47] Wolff SE, de Wit C. Intact endothelium-dependent dilation and conducted
responses in resistance vessels of hypercholesterolemic mice in vivo. J Vasc

[48] Larsen BT, Campbell WB, Gutterman DD. Beyond vasodilatation: non-
vasoctor role of epoxyeicosatrienoic acids in the cardiovascular system. Trends

primary metabolite of nicotineamide, exerts anti-thrombotic activity medi-
ated by cyclooxygenase-2/prostacyclin pathway. Br J Pharmacol 2007;152:
230–9.