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Functional alterations in endothelial NO, PGI_2 and EDHF pathways in aorta in ApoE/LDLR$^{-/-}$ mice

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ABSTRACT

Adequate endothelial production of nitric oxide (NO), endothelium-derived hyperpolarizing factor (EDHF), and prostacyclin (PGI_2) 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 PGI_2-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)/PGI_2 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/PGI_2 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/PGI_2 and EDHF pathways may compensate for the loss of the biological activity of NO.

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

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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 (PGI_2) and endothelium-derived hyperpolarizing factor (EDHF) [2]. Since the contribution of PGI_2 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 PGI_2 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 PGI_2 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, PGI_2 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 PGI_2 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 Comittee 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 ventricule. Plasma was separated by centrifugation at 1000 × 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 Camedia 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 (mm^2) 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