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Comparison between Sexes during the Development of Atherosclerosis in apoE & eNOS: Double Knockout Mice

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Abstract

The aim of the study was to investigate differences between sexes in time – dependent development of atherosclerosis in apoE & eNOS – double knockout mice. At four time points, groups of female and male mice were sacrificed and the size of atherosclerosis was measured by "cross section". At the age of 2 months, there was no difference between males and females. However, at the age of 4 months, the atherosclerosis was significantly bigger in females than in males (55,616 ± 4,622 μm^2 vs. 37,181 ± 4,142 μm^2; $p<0.05$). At the age of 6 months, the differences were even more pronounced (314,465 ± 19,351 μm^2 vs. 108,277 ± 24,549 μm^2; $p<0.001$). The same tendency was present at the age of 12 months (488,356 ± 49,823 μm^2 vs. 201,646 ± 43,886 μm^2; $p<0.001$). We describe for the first time the development of atherosclerosis in both sexes of apoE & eNOS – double knockout mice, proving that as early as at age of 16 weeks, a substantial difference in the size of atherosclerosis is noted between females and males, in favour of the females. This difference becomes more significant with the age of mice. The possible mechanisms responsible for such differences require further investigation.

Keywords: Atherosclerosis; Gene-targeting; Sex difference

Introduction

Nitric oxide (NO), released by the vascular endothelium in response to various stimuli, including acetylcholine and shear stress of blood flow plays an important role in endothelium-dependent vasodilation [1-3]. It was shown, that in patients with coronary heart disease arteries constrict, rather than dilate, in response to the acetylcholine, in a phenomenon called endothelial dysfunction [4]. Several molecular mechanisms have been proposed to explain such phenomenon, namely deficiencies in substrate (L-arginine) or cofactors (e.g., tetrahydrobiopterin) for nitric oxide synthases (NOS), alterations in membrane signalling, or enhanced, oxidative stress-dependent degradation of NO [5].

Importantly, besides the effects on vascular tone, NO shows other physiological activities, relevant to its antiatherogenic actions including the inhibition of smooth muscle cell proliferation, prevention of platelet aggregation, as well as inhibition of leukocyte activation and adhesion [6-9]. Therefore, to study the interactions between eNOS and atherogenesis mouse models of atherosclerosis, such as apolipoprotein E (apoE) knockout (KO) mice [10,11], have been combined with models of endothelial nitric oxide synthase (eNOS) deficiency. It was demonstrated that mice lacking both eNOS and apoE had significantly increased blood pressure, developed larger atherosclerotic plaques and had more severe kidney damage than apoE-deficient mice with an intact eNOS function [12-15].

Interestingly, apoE-KO mice develop gender specific differences in the development of atherosclerotic changes in aorta, which typically occur earlier and are bigger in female mice [16]. The reasons of this have never been fully elucidated, however, apoE-KO mice have been reported to show intriguing sex differences in the production of important endothelial mediators, namely prostacyclin (PGI_2) and thromboxane A_2 (TXA_2) [17]. As many mutual influences between vascular PGI_2 and NO generation have been recognized [18,19] and several mechanisms responsible for controlling NO generation seem to be gender-specific [20,21] we aimed our study to investigate whether the lack of eNOS-derived NO could influence the sex-related differences in development of atherosclerosis in apoE-KO mice. Therefore we compared the formation and structure of atherosclerotic plaques in female and male apoE & eNOS – double knockout (DKO) mice.

Materials and Methods

Animals and procedures

ApoE & eNOS – double knockout mice on B6.129P2 background were created from apoE-knockout and eNOS-knockout mice by Jackson Laboratory (Bar Harbor, Maine, USA) (project number 21536_BHSM). The apoE and eNOS PCR genotyping was performed according to company protocols (http://jaxmice.jax.org/protocolsdb/) (Figure 1). The mice were maintained on 12 h dark / 12 h light cycles in air-conditioned rooms (22.5 ± 0.5°C, 50 ± 5% humidity), with not constraint access to food and water. The mice were put on chow diet made by Wytwornia Pasz Morawski (Kcynia, Poland). Then, at different four ages (2 months, 4 months, 6 months and 12 months), groups (n=4 each) of female and male mice were injected with 1000 IU of fraxiparine (Sanofi-Synthelabo, France) into the peritoneum, then were killed using a carbon dioxide chamber.

The blood was collected from the right ventricle. The right atrium was incised and the heart was perfused by PBS through the apex of the left ventricle, at a constant pressure of 100 mmHg, next, the heart was dissected [22,23]. All animal procedures were approved by the Jagiellonian University Ethical Committee on Animal Experiments.

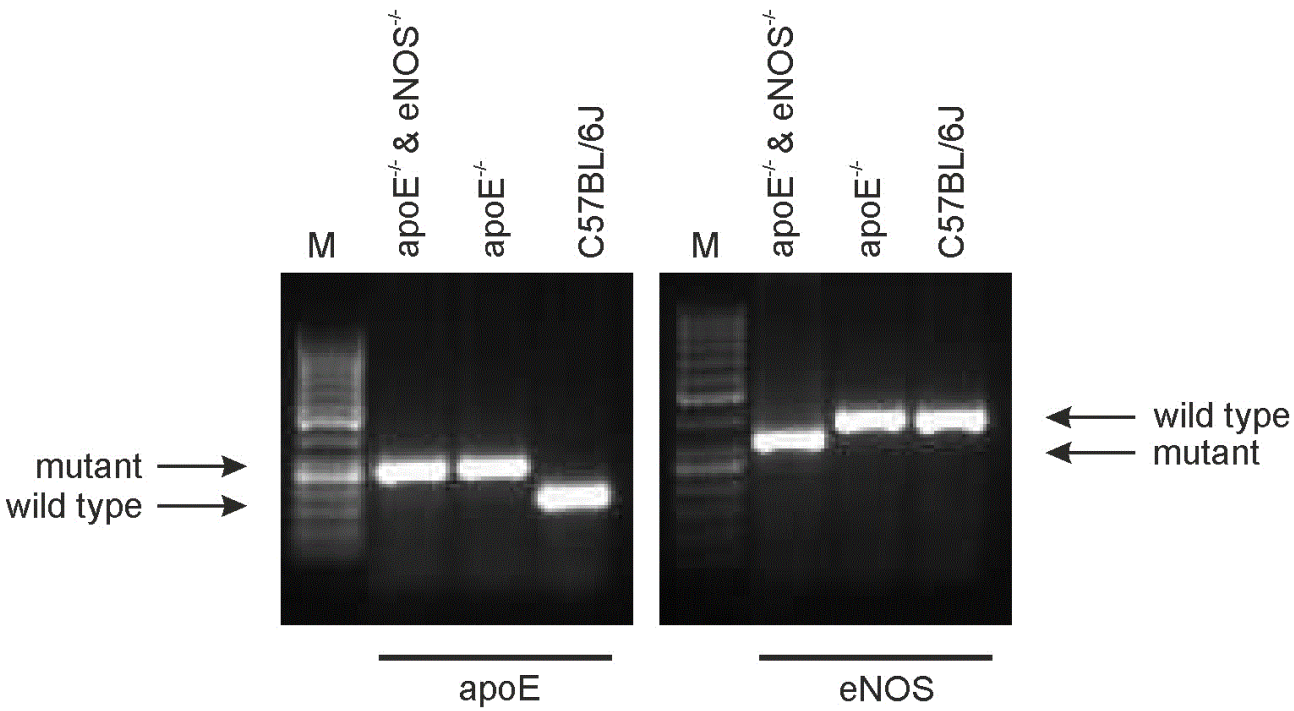

Figure 1: PCR genotyping of apoE & eNOS – double knockout mice. The left band shows PCR products characteristic for ApoE–/– (245 bp) and wild-type (155 bp) mice. The right band shows PCR products for eNOS–/– (258 bp) and wild-type (337 bp) mice. M – marker.

Quantification of atherosclerosis

The heart with the ascending aorta were embedded in OCT compound (CellPath, UK) and frozen immediately. 10 μm thick serial sections were cut from the proximal 1 mm of the aortic root using a standardized procedure [24,25]. For each heart, nine adjacent sections were collected at 100 μm intervals starting at a 100 μm distance from the appearance of the aortic valves. Then sections were fixed in 4% paraformaldehyde, stained Oil red-O (Sigma-Aldrich, USA) and examined under an Olympus BX50 (Olympus, Tokyo, Japan) microscope. Pictures were taken using an Olympus Camedia DP71 digital camera. Total area of the lesion was measured in each slide using LSM Image Browser 3 software (Zeiss, Jena, Germany). For each mouse, a mean lesion area was calculated from nine sections what reflects the cross-section area covered by atherosclerosis. The same procedure was performed with Sirius red stained sections for collagen measurements.

Immunohistochemistry staining of aortic roots

Sections were pre-incubated with 5% non-immunogenic goat serum, with 2% fat-free milk, to block nonspecific binding of antibodies (Abs). Incubations with primary antibodies: anti-α-smooth muscle actin FITC-conjugated (α-SMA; dilution 1:800; Sigma-Aldrich) and rat anti-mouse CD68 (dilution 1:800; Serotec, Kidlington, UK) were performed overnight at room temperature in wet chambers. Afterwards, goat anti-rat Cy3-conjugated antiserum (Jackson ImmunoResearch, West Grove, PA) was diluted 1:800 and applied to visualize rat Abs. Sections were analyzed using a fluorescence Olympus BX50 microscope containing appropriate filter cubes to show Cy3 (red) and FITC (green) fluorescence. The pictures were taken with Olympus Camedia DP71 digital camera. The total area occupied by CD68-immunopositive macrophages and α-SMA-positive cells in each section was measured using LSM Image Browser 3 software [26].

Plasma lipids

The plasma was separated by centrifugation at 1000×g at 4°C for 10 min and stored in –80° C until used. Total cholesterol, HDL-cholesterol, LDL-cholesterol and triglycerides were measured with commercially available kits (Roche Molecular Biochemical, USA).

Statistical analysis

The results are presented as mean ± SEM. The nonparametric Mann-Whitney U test (cross section, Sirius red and IHC data) or t-test (plasma lipidogram) were used for analysing the data. $P<0.05$ is considered to be statistically significant.

Results

Oil red-O staining showed that females apoE & eNOS – double knockout mice developed larger atherosclerotic lesions compared to males. Differences between females and males appeared for the first time at the age of 4 months: 59,808 ± 4,192 μm^2 vs. 41,325 ± 4,143 μm^2; $p<0.05$. At the age of 6 months, the differences were even more pronounced: 314,465 ± 19,351 μm^2 vs. 108,277 ± 24,549 μm^2; $p<0.001$. The same tendency was noted at the age of 12 months: 488,356 ± 49,823 μm^2 vs. 201,646 ± 43,886 μm^2; $p<0.001$ (Figures 2A and 2C).

Similarly, the total collagen content, determined by Sirius red staining, was higher in females compared to males. At the age of 4 months, the collagen area was 49,110 ± 2,890 μm^2 in females and

39,228 ± 5,989 μm^2 in males. At the age of 6 and 12 months, the differences were bigger: 319,277 ± 5,722 μm^2 vs. 110,608 ± 22,312 μm^2 and 496,497 ± 55,474 μm^2 vs. 186, 242 ± 46,463 μm^2; $p<0.05$, respectively (Figures 2B and 2D).

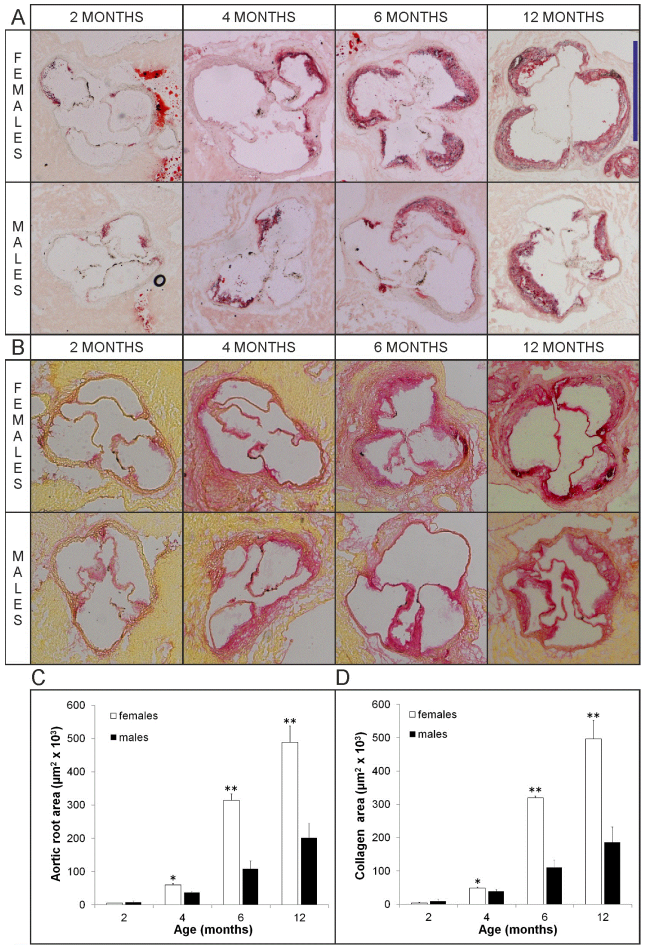

Figure 2: Representative micrographs showing Oil-red O stained (A) and Sirius red stained (C) lesions at different ages (2, 4, 6 and 12 months) in apoE & eNOS – double knockout females and males (magnification × 40). Atherosclerotic lesion area measurements (B) and collagen area measurements (D) in the aortic root at four different ages in apoE & eNOS – double knockout mice in females comparing to males. Mean ± SEM, n=4, *p<0.05; **p<0.001. The scale bar represents 1 mm.

There were also significant differences at the age of 6 and 12 months between females and males in macrophages-occupied areas in the lesion or in the content of smooth muscle cells in the fibromuscular cap. The plaques in female 6 and 12 months old mice contained significantly more macrophages and smooth muscle cells; however, the composition of plaques (the ratio of CD68 to α-SMA) was not significantly different between the sexes (Figure 3).

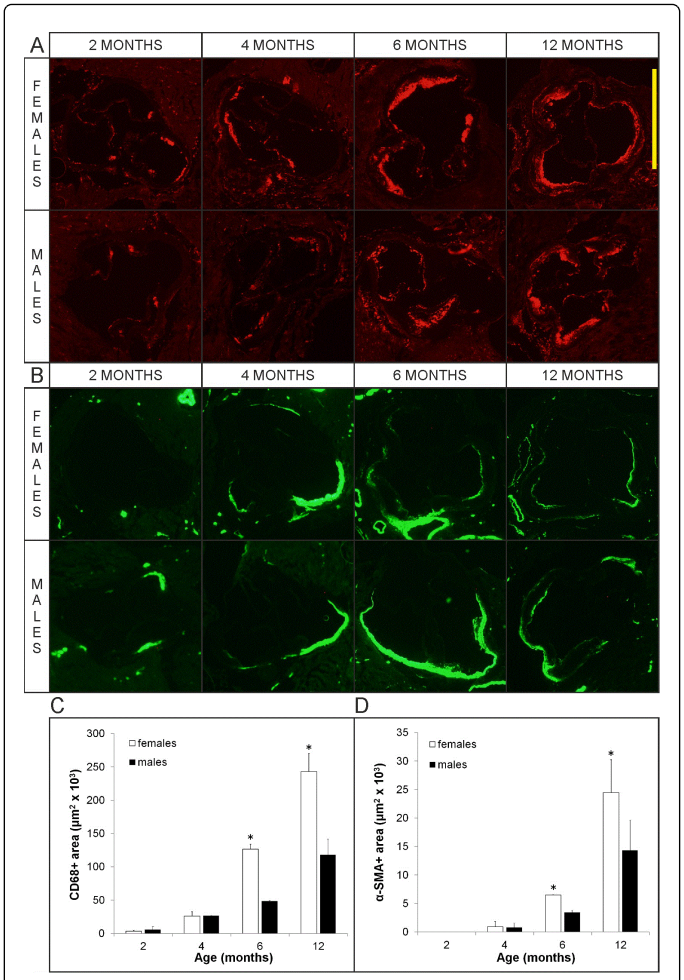

Figure 3: Representative immunohistochemical staining for macrophages marker CD68 (A) and α-actin of smooth muscle (SMA) (C) at four different ages (2, 4, 6 and 12 months) in apoE & eNOS – double knockout females and males. Quantitative analysis of CD68-positive macrophages (B) and SMA-positive smooth muscle cells (D) at four different ages in apoE & eNOS – double knockout mice in females, comparing to males. Mean ± SEM, n=4, *p<0.05. The scale bar represents 1 mm.

The levels of total cholesterol, HDL-cholesterol, LDL-cholesterol, TGs in blood did not differ significantly between the sexes (Table 1).

Age		TCH (mmol/L)	HDL (mmol/L)	LDL (mmol/L)	TG (mmol/L)
2 months	♀	11.25 ± 1.76	4.6 ± 1.91	8.45 ± 1.35	1.03 ± 0.05
	♂	10.85 ± 1.76	2.45 ± 0.05	6.35 ± 0.65	2.29 ± 0.67
4 months	♀	12.9 ± 1.6	2.65 ± 0.05	8.8 ± 1.0	1.23 ± 0.02
	♂	13.85 ± 1.45	2.65 ± 0.25	9.6 ± 0.5	1.47 ± 0.13
6 months	♀	13.9 ± 3.21	4.1 ± 1.5	10.25 ± 2.06	0.97 ± 0.03
	♂	16.3 ± 2.31	2.7 ± 0.1	10.35 ± 1.76	1.86 ± 0.04

12 months	♀	12.7 ± 0.3	4.5 ± 1.5	10.00 ± 1.2	0.66 ± 0.07
	♂	11.5 ± 0.8	5.0 ± 2.31	8.75 ± 0.85	1.02 ± 0.06

Table 1: Plasma level of total cholesterol, high-density Lipoproteins (HDL), low-density lipoproteins (LDL) and triglycerides (TG) at four different ages in apoE & eNOS – double knockout mice in females and males.

Discussion

The antiatherosclerotic properties of endothelium-derived NO are widely recognized [27] – e.g., its direct inhibitory action on vascular smooth muscle cell proliferation has been described in eNOS–/– mice [28,29]. Not surprisingly, the enhancement of activity of endothelial isoform of nitric oxide synthase (eNOS) represents an interesting target for the prevention or therapy of cardiovascular diseases [30]. Also recently, using this model we proved directly the necessity of the presence of eNOS in endothelium for nebivolol to show its anti-atherogenic potency [31]. On the other hand, it has been shown that pharmacological inhibition of eNOS accelerated atherosclerosis in rabbits [32] and in apoE-knockout mice [33]. In line with such observations, it has been reported that the atherosclerotic changes were shown to be more pronounced in apoE & eNOS – double knockout mice as compared to single apoE-KOs [15,34]. Here, for the first time we comprehensively describe the development of atherosclerotic changes in both sexes of apoE & eNOS – double knockout mice, proving that starting from the 16th week on, a substantial sex-difference in size of plaques in aorta was noted in favour of the female mice. Noteworthy, in our study the composition of plaques (the ratio of CD68 to α-SMA) remained the same between sexes. Our results are consistent with those reported by Knowles et al. [14], showing that at single time point of 4 months of age the atherosclerotic plaques measured by the cross-section method in the proximal aorta of female apoE & eNOS – double knockout mice were significantly larger than in male animals. In contrast, Kuhlencordt et al. have not seen differences between the sexes in this model [15,34]. The authors explained the differences between their results and those obtained by Knowles et al. [14], by feeding mice with a high-cholesterol diet and by using much less sensitive method of size estimation of atherosclerosis (en face instead of cross section). Importantly, in our study, atherosclerosis was assessed by the cross section method and the mice were fed by chow diet.

The sex-differences in development of atherosclerotic plaques in aortas of apoE-KO mice are documented quite extensively. Caligiuri et al. [16] described for the first time that female apoE-knockout animals had significantly greater atherosclerosis than male apoE-knockout mice. She proved that atherosclerotic lesions were larger and also more advanced in young female than in male "gene-targeted" mice. At the beginning, it was a surprising observation, since in humans the situation is the opposite. However, soon the findings of Caligiuri et al. were confirmed in many publications [17,35-40]. Our study shows, that similar sex-differences in development of atherosclerotic plaques could be observed in apoE & eNOS – double knockout mice. The question arises about the possible causes of sex-dependent differences in the development of atherosclerosis in mice models. Despite the long-term use in research of apoE-KO mice, the reasons of this somehow surprising phenomenon, regarding in general opposite tendency in humans, have never been fully elucidated. Apparently, the differences in plasma lipids could not be the cause – in the majority of studies the average levels of total cholesterol, LDL-cholesterol, HDL-cholesterol and triglycerides did not differ significantly between male and female apoE-KO mice; in one study, the plasma levels of total cholesterol were even lower in female mice than in males despite more pronounced atherosclerosis [17]. Importantly, in our hands lipid levels did not differ between male and female apoE & eNOS – double knockout mice either.

Smith et al. described intriguing differences in the production of antiatherogenic prostacyclin (PGI_2) vs. proatherogenic thromboxane (TXA_2): apoE-KO females fed on high-fat diet produced up to 15-fold higher TXA_2 and 50% lower PGI_2 than males, as evidenced by urine excretion of these prostanoids [17]. The authors speculate that such a shift in the equilibrium between TXA_2 and PGI_2 constitutes a proatherogenic risk factor in female apoE-KO mice. This could be a case in apoE & eNOS – double knockout mice, as our preliminary measurements of plasma levels of stable metabolites of TXA_2 and PGI_2 (TXB_2 and 6-keto $PGF_{1\alpha}$, respectively) showed the same pattern (data not shown). However, still, the molecular mechanisms responsible for such sex-dependent differences in TXA_2 and PGI_2 generation either in apoE-KO or in apoE & eNOS – double knockout remain unknown. We cannot translate directly our results to humans. However, it forms an important piece of information about precious, but very fragile apoE & eNOS – double knockout mice model of atherosclerosis. There are already only 6 publications describing this model, in comparison to hundreds articles about apoE-knockout or LDLR-knockout mice. To sum up, we have compared the development of atherosclerosis in both sexes of apoE & eNOS – double knockout mice in several time points, proving that from 16th week on, a substantial difference could be noted in the size, but not in the composition of atherosclerotic changes in aorta between females and males, in favour of the females. The possible mechanisms responsible for such differences require further investigation.

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