INTRODUCTION

Since 1992 the mouse has become an excellent model for experimental atherosclerosis research. Until 1992, the diet-induced atherosclerosis mouse model has been used effectively, but the lesions tended to be small and were limited to early fatty-streak stage. In 1992 the first line of gene targeted animal models, namely apolipoprotein E (apoE)-single knockout mice was developed. Of the genetically engineered models, the apoE-deficient model was the only one that developed extensive atherosclerotic lesions on a chow diet. The creation of apoE-single knockout mice has changed the face of atherosclerosis research (1).

In 2001, more sophisticated model of atherogenesis: apoE and endothelial nitric oxide synthase (eNOS)-double knockout mice has been created independently in two laboratories (2, 3). It has shown that chronic deficiency of eNOS increases atherosclerosis in apoE-knockout mouse model. Furthermore, in the absence of eNOS, peripheral coronary disease, chronic myocardial ischemia, heart failure, and an array of vascular complications develop that have not been observed in apoE-knockout animals. However, soon it has occurred that there was a big problem with the model: apoE and eNOS-double knockout mice bred extremely poor. In spite of this (since the model is excellent), we decided that after our experiments with nebivolol in apoE-single knockout mice (4, 5), we would try to use apoE and eNOS-double knockout mice to investigate the mechanism of nebivolol anti-atherogenic action.

MATERIALS AND METHODS

Animals and treatment

All animal procedures were approved by the Jagiellonian University Ethical Committee on Animal Experiments.

Ten female apoE and 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). Thirteen female apoE-single knockout mice on B6.129P2 background were purchased from Jackson Laboratory (Bar Harbor, Maine, USA). Mice were maintained on 12-h dark/12-h light cycles in air-conditioned rooms (22.5 ± 0.5°C, 50 ± 5% humidity) and access to diet and water ad libitum. At the age of 8 weeks mice were put on chow diet made by Ssniff (Soest, Germany) for 4 months. The comparison between apoE-single knockout mice and apoE & eNOS-double knockout mice without treatment also showed statistically significant difference: 81,232 ± 8,264 µm² versus 92,319 ± 8,876 µm² (P<0.05). This is the first report that describes the effect of nebivolol on atherogenesis in apoE and eNOS-double knockout mice, proving directly the necessity of the presence of eNOS in endothelium for nebivolol to show its an anti-atherogenic potency.
Therefore, we received four groups: A: control apoE-single knockout (n=7); B: apoE-single knockout treated with nebivolol (n=6); C: control apoE and eNOS-double knockout (n=5); D: apoE and eNOS-double knockout treated with nebivolol (n=5).

Procedures

At the age of 6 months mice were sacrificed under anesthesia and 1000 UI of fraxiparine (Sanofi-Synthelabo, France) was injected into the peritoneum. The blood was collected from the right ventricle. Plasma was separated by centrifugation at 1000 \times g at 4°C for 10 min and stored in –80°C. Then, right atrium was incised and the heart was perfused by PBS through the apex of the left ventricle at a constant pressure of 100 mm Hg. Next, the heart was dissected (6, 7).

Plasma lipids

Total cholesterol and triglycerides were assayed using commercially available kits (Roche Molecular Biochemical, USA).

Quantitation of atherosclerosis

The heart and ascending aorta were embedded in OCT compound (CellPath, UK) and snap-frozen. Ten micrometer-thick cryosections were cut from the aortic root using a standardized protocol (8, 9).

<table>
<thead>
<tr>
<th>group</th>
<th>TCH (mmol/l)</th>
<th>HDL (mmol/l)</th>
<th>LDL (mmol/l)</th>
<th>TG (mmol/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>9.45</td>
<td>4.98</td>
<td>6.05</td>
<td>0.73</td>
</tr>
<tr>
<td>B</td>
<td>9.53</td>
<td>5.88</td>
<td>6.15</td>
<td>0.75</td>
</tr>
<tr>
<td>C</td>
<td>15.4*</td>
<td>8.08*</td>
<td>11.58*</td>
<td>0.99*</td>
</tr>
<tr>
<td>D</td>
<td>13.45*</td>
<td>6.15*</td>
<td>9.45*</td>
<td>1.22*</td>
</tr>
</tbody>
</table>

Table 1. Total cholesterol (TCH), HDL-cholesterol, LDL-cholesterol and triglycerides (TG) levels in groups A-D, presented as mean. *P<0.05 compared to group A and B.

RESULTS

Nebivolol did not change the level of cholesterol and triglycerides in blood, as compared to the control group, both in apoE-single knockout as well as in apoE and eNOS-double knockout mice.

Serial sections were cut from the proximal 1 mm of the aortic root. Eight adjacent sections were collected at 100-µm intervals starting at a 100-µm distance from the appearance of the aortic valves. Sections were thaw-mounted on poly-L-lysine coated slides and air dried. After fixation in 4% paraformaldehyde (pH = 7), sections were stained with Meyer's hematoxylin and oil red-O (Sigma-Aldrich, USA). Oil red O-stained sections were examined under Olympus BX50 (Olympus, Tokyo, Japan) microscope and used for quantitative evaluation. Images of the aorta were recorded using Olympus Camedia 5050 digital camera and stored as TIFF files of resolution 1024 × 768 pixels. Total area of the lesion was measured semiautomatically in each slide using LSM Image Browser 3 software (Zeiss, Jena, Germany). For each animal a mean lesion area was calculated from eight sections, reflecting the cross-section area covered by atherosclerosis.

Results are expressed as mean ± S.E.M. The nonparametric Mann-Whitney U test was used for analysis of the data. P<0.05 was considered as statistically significant.

Fig. 1. Atherosclerostic lesions, presented as mean in (A) control apoE-single knockout mice, (B) nebivolol-treated apoE-single knockout mice, (C) control apoE and eNOS-double knockout mice and (D) nebivolol-treated apoE and eNOS-double knockout mice. *P<0.05; NS - non statistically significant difference between groups.
knockout groups. However, lipid level was significantly higher in double knockout mice (Table 1).

In apoE-single knockout mice, lesion area measured by "cross-section" of aortic roots was 79,244 ± 6,143 µm$^2$ in the control group versus 65,347 ± 6,152 µm$^2$ in nebivolol-treated group (P<0.05).

In apoE & eNOS-double knockout mice, lesion area measured by "cross-section" of aortic roots was 92,319 ± 8,876 µm$^2$ in the control group versus 98,609 ± 9,164 µm$^2$ in nebivolol-treated group (P<0.05).

The comparison between apoE-single knockout mice and apoE and eNOS-double knockout mice without treatment, showed statistically significant difference: 81,232 ± 8,264 µm$^2$ versus 92,319 ± 8,876 µm$^2$ (P<0.05) (Fig. 1 and 2).

**DISCUSSION**

Nebivolol is a third-generation β-blocker with vasorelaxation properties (10). It has been suggested that this effect is mediated by increased nitric oxide (NO) production, because it can be abrogated by inhibitors of NO synthase (NOS) (11). In vivo metabolized nebivolol increases vascular NO production. This phenomenon involves endothelial β2-adrenergic receptor ligation, with a subsequent rise in endothelial free [Ca$^{2+}$]i and endothelial NO synthase-dependent NO production. This may be an important mechanism underlying the nebivolol-induced, NO-mediated arterial dilation in humans.

Dessy et al. identified β3-adrenoreceptors in the endothelium of human coronary resistance microarteries, where they mediate an endothelium-dependent relaxation to both endogenous catecholamines and 3-adrenoreceptor-preferential agonists (12).

The expression of β3-adrenoreceptor mRNA and protein was demonstrated in extracts of human coronary microarteries. Immunohistochemical analysis revealed their exclusive localization in the endothelium, with no staining of vascular smooth muscle. In contractility experiments in which videomicroscopy was used, the nonspecific β agonist isoproterenol and the β3-preferential agonist BRL37344 evoked an 50% relaxation of endothelin-1 - preconstricted human coronary microarteries. Relaxations were blocked by the 1/2/3-adrenoreceptor antagonist bupranolol, but were insensitive to the 1/2-adrenoreceptor antagonist nadolol, confirming a β3-adrenoreceptor-mediated pathway.

Dessy et al. also showed this response to be mediated through the production of both NO and a hyperpolarizing factor that partly maintains vessel relaxation when eNOS is inhibited. Such a dual mechanism would be particularly suitable in circumstances of reduced NOS activity or NO bioavailability, as commonly found in atherosclerotic and ischemic diseases (12).

They, therefore, examined also whether nebivolol could activate β3-adrenoreceptors to mediate NO-dependent vasodilatory effects in isolated human and rodent coronary microvessels and characterized the transduction pathway leading to eNOS activation (13). Moreover, comparative use of rings from β3-AR(−/−) and eNOS(−/−) mice again confirmed that this property depends on the presence of both eNOS and β3-adrenoreceptors.

Hypertension and atherosclerosis are major factors in the etiology of ischemic heart disease and cerebrovascular disease - the 2 leading causes of death worldwide - and are important in the development of kidney dysfunction, congestive heart failure, and angina (2).

Considerable epidemiological evidence suggests that high blood pressure (BP) may have a direct role in enhancing atherosclerotic lesion formation (14). Atherosclerosis is 3 times more common in patients with hypertension, and there is a positive, although not linear, correlation between BP and atherosclerosis. In addition, atheroma formation occurs in muscular arteries but not in lower-pressure veins, and hypertension promotes lesion formation in the presence of hypercholesterolemia (14). Clinically, many antihypertensive drugs are effective in reducing morbidity and mortality from atherosclerotically mediated cardiovascular events (15). Despite this body of evidence, the effect of hypertension on atherosclerosis has been difficult to study in humans because of confounding variables and the complexity of the genetics underlying each condition. Animal studies have been equally hindered by the lack of appropriate models with simultaneous genetic predispositions for atherosclerosis and hypertension.

In 2000, Knowles et al. created for the first time such a model, generated by breeding mice that spontaneously develop atherosclerosis due to apoE-deficiency with mice that are hypertensive due to lack of the endothelial nitric oxide synthase gene (eNOS-knockout) (2).

The eNOS serves important basal regulatory functions in the vasculature. In response to stimuli such as shear stress or acetylcholine, eNOS catalyzes the production of nitric oxide (NO) from L-arginine. The NO diffuses across the endothelial cell membrane into neighboring smooth muscle cells and induces vasodilation. NO also acts locally to prevent platelet and leukocyte aggregation and inhibits vascular smooth muscle cell proliferation (16). Direct evidence that eNOS mutations can
cause hypertension has been presented by Shesely et al. (17) and Huang et al. (18), who showed that mice lacking eNOS have increased BP, decreased heart rate, and increased plasma renin activity, but no atherosclerosis. Although linkage between genetic polymorphisms in the eNOS gene and essential hypertension has not been conclusively documented in humans, there is substantial evidence that NO pathways are disrupted in both hypertension and atherosclerosis (19, 20).

Knowles et al. demonstrated that mice lacking both eNOS and apoE have significantly increased BP, develop larger plaques, and have more severe kidney damage than do apoE-deficient mice with intact eNOS function (2). The effects were ameliorated by treatment with the angiotensin-converting enzyme (ACE) inhibitor enalapril. However, Chen et al. in 2001 showed that the effects of eNOS gene deficiency on accelerating atherogenesis are not solely due to hypertension, in that lowering the blood pressure to normal in the apoE and eNOS-double knockout mice did not reduce the lesion area (21).

Besides the effects on BP and heart rate, the eNOS system is potentially antiatherogenic through many other mechanisms. For example, a direct role for NO in decreasing vascular smooth muscle cell proliferation after a remodeling stimulus has been described in eNOS-knockout mice (22). In addition, NO inhibition of platelet aggregation and monocyte adherence to the vessel wall are potentially antiatherogenic (16, 19), and antioxidant functions of NO may prevent proatherogenic changes in lipoproteins (23). Many of the local functions of NO counterbalance proatherogenic effects of angiotensin II, which mediates vasoconstriction, smooth muscle cell migration and proliferation, and monocyte adhesion. It is important to note that eNOS-deficient mice have increased plasma renin activity (17), and probably also have increased production of angiotensin II.

Moreover, Kuhlenordt et al. described that mice total cholesterol, and lipoprotein profile did not differ between apoE-single knockout mice and apoE and eNOS-double knockout mice apoE/eNOS-DKO mice fed the Western-type diet for 16 weeks. This is in contradiction to our observations. However, in our experiment we fed mice only by chow diet, which can explain the differences.

Anti-atherogenic action of nebivolol, first shown in rabbit model of atherosclerosis (13, 14) and than confirmed in apoE-single knockout mouse model (4, 24), can be explained by its beneficial effect on endothelium. We described also that nebivolol suppresses the inflammatory processes in the plaque and enhances its stability (5). The results of our experiment confirmed the role of eNOS in the action of nebivolol. In apoE-single knockout mice treated with nebivolol (group B) the atherosclerosis was statistically significant decreased by the action of nebivolol, compared to apoE-single knockout control (group A). However, that was not the case in apoE & eNOS-double knockout mice treated with nebivolol (group D), compared to apoE and eNOS-double knockout control (group C) (Fig. 1 and 2). Moreover, as was described in earlier articles (2, 3, 21, 25-27), total atherosclerosis in apoE and eNOS-double knockout mice is bigger than in apoE-single knockout mice.

Our results show clearly that only in the presence of eNOS, nebivolol significantly decreases atherogenesis. Although this finding confirms the conclusion drawn previously, here, thanks to the use of eNOS knockout animals, our evidences gain a strong direct character which is reflected in the fact that the knockout of eNOS gene is able to destroy the anti-atherosclerotic effect of nebivolol. Nebivolol, but not atenolol, is known to reverse endothelial dysfunction in arterial hypertension both in experimental models as well as in patients (28, 29). Moreover, nebivolol prevents vascular nitric oxide synthase (NOS) III uncoupling in experimental hyperlipidemia and inhibits NADPH oxidase activity in inflammatory cells (30).

Of note, despite our plans, we were able to receive only 10 female apoE and eNOS-double knockout mice. It was caused by extremely poor breeding of such animals. One can see that until now worldwide, there are only four articles available about apoE and eNOS-double knockout mice, comparing to thousands, relating to apoE-knockout. Naturally, so poor breeding of double knockout mice strongly limited our previous plans, including the number of experiments as well as our plans on molecular studies. However, despite this, hoping that our results may open new area of research on atherogenesis and the mechanism of anti-atherogenic activity of nebivolol, we have decided to submit our results in the state in which we managed to achieve till now.

To our knowledge, this is the first report that describes the effect of nebivolol on atherogenesis in apoE and eNOS-double knockout mice, proving directly the necessity of the presence of eNOS for nebivolol to show an anti-atherogenic potency.

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**Conflict of interests:** None declared.

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