The effect of AVE 0991, nebivolol and doxycycline on inflammatory mediators in an apoE-*knockout* mouse model of atherosclerosis

Authors' Contribution:
A Study Design
B Data Collection
C Statistical Analysis
D Data Interpretation
E Manuscript Preparation
F Literature Search
G Funds Collection

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Summary

Background: The aim of this study was to investigate whether the 3 different substances that can decrease the development of atherosclerosis – nebivolol, AVE 0991 and doxycycline – could at the same time diminish the level of inflammatory indicators interleukin-6 (IL-6), interleukin-12 (IL-12), serum amyloid A (SAA), and monocyte chemotactic protein-1 (MCP-1).

Material/Methods: Forty 8-week-old female apoE–knockout mice on the C57BL/6J background were divided into 4 groups and put on chow diet for 4 months. Three experimental groups received the same diet as a control group, mixed with AVE 0991 at a dose 0.58 µmol per kg of body weight per day, nebivolol at a dose 2.0 µmol per kg of body weight per day, and doxycycline at a dose 1.5 mg per kg of body weight per day. At the age of 6 months, the mice were sacrificed.

Results: All inflammatory indicators (MCP-1, IL-6, IL-12 and SAA) were diminished by AVE 0991. There was also a tendency to lower MCP-1, IL-6, IL-12 and SAA levels by nebivolol and doxycycline; however, it did not reach statistical significance.

Conclusions: Of the 3 presented substances, only AVE 0991 was able to diminish the rise of inflammatory markers. Therefore, drug manipulations in the renin-angiotensin-aldosterone axis seem to be the most promising in the future treatment of atherogenesis.

key words: atherosclerosis model • inflammatory markers • nebivolol • AVE 0991 • doxycycline

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BACKGROUND

Although atherosclerosis was long thought to be mainly a degenerative disease, it is now well ascertained that its pathogenesis is inflammatory. The pivotal stage of atherogenesis is the antigen presentation by macrophages to T lymphocytes. This antigen could be a fragment of oxidized low-density lipoproteins "digested" by macrophages, heat shock protein 60, β2-glycoprotein I, or fragments of bacterial antigens. For interaction between the immunological cells, the presence of CD40 receptor on macrophages and its ligand CD40L on the surface of T lymphocytes are necessary. During the interaction between these cells an immunological type T helper 1 or Treg response arises. Th1 response and its mediators – interferon γ, tumor necrosis factor α, interleukin-1, interleukin-12 and interleukin-18 – enhance atherogenesis, whereas Treg response and its mediators – interleukin-10 and transforming growth factor β (TGF-β) – inhibit the development of atherosclerosis [1].

apoE-*knockout* mice played an important role in the history of a new theory of atherogenesis since there has been lack of unequivocal evidence of an important inflammatory component in atherogenesis. This evidence was delivered by a new technique – gene targeting – for the invention of which Mario R. Capecchi, Martin J. Evans and Oliver Smithies received the Nobel Prize in Physiology or Medicine in 2007.

This technique enabled the investigators to create apoE-*knockout* mice [2,3], an ideal animal model to test the influence of singular proteins participating in the inflammatory response on the development of atherosclerosis. These studies showed, for example, that the absence of only 1 cytokine, interferon γ (IFN-γ), reduced atherosclerosis by 60% [4]. The overexpression of adhesive molecules (vascular adhesion molecule 1 and intercellular adhesion molecule 1) at sites with atheromatous changes was also observed in apoE-*knockout* mice [5]. Monocyte chemotactic protein (MCP-1) was shown to play an important part in the progression of atheromatous lesions [6,7]. Moreover, it was observed that interleukin-18 knockout decreased atherosclerosis by 35% [8,9].

Finally, in apoE-*knockout* mice with severe combined immunodeficiency (SCID), atherosclerosis was reduced by 70% in comparison to the control group, due to a significantly lower number of lymphocytes in mice with SCID. It was demonstrated that transfer of T cells to these mice aggravated atherosclerosis by 164% [10].

Atherosclerosis is therefore a chronic inflammatory disease, in most cases initiated by hypercholesterolemia. Nowadays, hypercholesterolemia and inflammation are considered as "partners in crime". The concept of atherosclerosis as inflammatory disease is fairly new, but it is already considered as an undisputable achievement of science and has particular therapeutic consequences [11–13].

Our goal was to investigate whether 3 different substances that can decrease the development of atherosclerosis – nebivolol, AVE 0991 and doxycycline [14–16] – could at the same time diminish the level of inflammatory indicators interleukin-6 (IL-6), interleukin-12 (IL-12), serum amyloid A (SAA), and monocyte chemotactic protein-1 (MCP-1).

MATERIAL AND METHODS

Animals

Forty female apoE-*knockout* mice on the C57BL/6J background were obtained from Taconic (Ejby, Denmark) [8]. 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 food and water *ad libitum*. All animal procedures were approved by the Jagiellonian University Ethics Committee on Animal Experiments (approval nr Zi/496/2009).

Protocol of studies

At the age of 8 weeks, mice were put on chow diet made by Ssniff (Soest, Germany) for 4 months. Three experimental groups (in each n=10) received the same diet as a control group, mixed with: AVE 0991 (a kind gift from Sanofi-Aventis Deutschland GmbH, Frankfurt am Main, Germany) at a dose 0.58 μmol per kg of body weight per day, or nebivolol (a kind gift from Janssen Pharmaceutica, Geel, Belgium) at a dose 2.0 μmol per kg of body weight per day, as well as doxycycline (Sigma Aldrich, St. Louis, MO, USA) at a dose 1.5 mg per kg of body weight per day [17–19].

At the age of 6 months, 1000 UI of Fraxiparine (Sanofi-Synthelabo, France) was injected i.p. and after 10 min mice were sacrificed in a chamber filled with carbon dioxide. 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 [20].

Measurement of inflammatory mediators

Plasma levels of interleukin-6 (IL-6), interleukin-12 (IL-12) and serum amyloid A (SAA) (all from R&D Systems, Minneapolis, MN, USA) and macrophage chemotactic protein-1 (MCP-1) (BioSource, Camarillo, CA, USA) were measured using ELISA test [21].

Statistical analysis

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

RESULTS

All inflammatory indicators: MCP-1, IL-6, IL-12 and SAA were diminished by AVE 0991. There was also a tendency to lower MCP-1, IL-6, IL-12 and SAA levels by nebivolol as well as by doxycycline; however, it did not reach statistical significance.

As regards MCP-1, the level was 78±9 pg/ml in the control group, 44±5 pg/ml (p<0.05) in the AVE 0991-treated group, 69±7 pg/ml (p>0.05) in the nebivolol-treated group, and 74±6 pg/ml (p>0.05) in the doxycycline-treated group.

As for IL-6, in control group the level was 792±76 pg/ml, in the AVE 0991-treated group it was 447±24 pg/ml (p<0.05), in the nebivolol-treated group it was 685±67 pg/ml (p>0.05), and in the doxycycline-treated group it was 714±69 pg/ml (p>0.05).
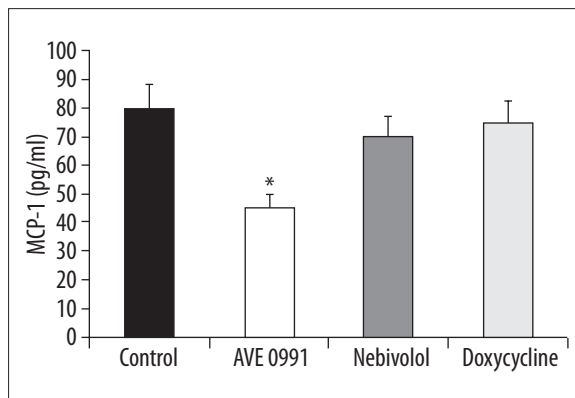
Figure 1. Plasma level of MCP-1 in all groups (n=10). * p<0.05, comparing to control group.
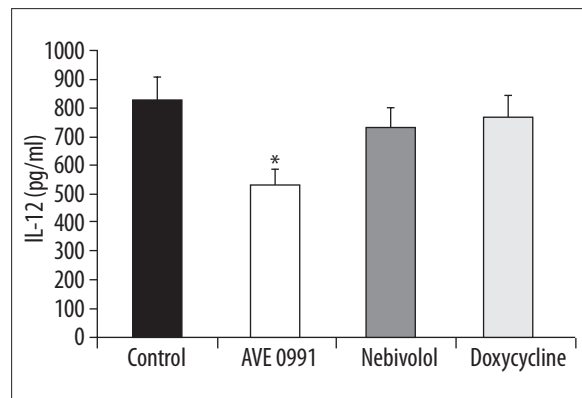

Figure 3. Plasma level of IL-12 in all groups (n=10). * p<0.05, comparing to control group.
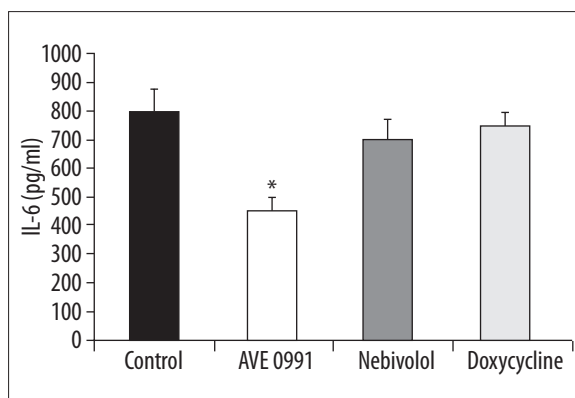
Figure 2. Plasma level of IL-6 in all groups (n=10). * p<0.05, comparing to control group.
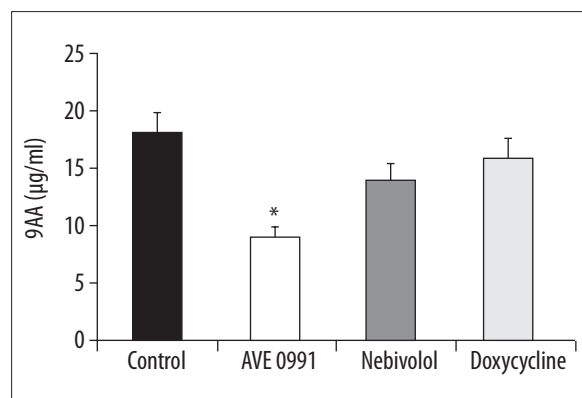

Figure 4. Plasma level of SAA in all groups (n=10). * p<0.05, comparing to control group.

IL-12: in the control group the level was 845±95 pg/ml, in the AVE 0991-treated group it was 524±65 pg/ml (p<0.05), in the nebivolol-treated group it was 736±67 pg/ml (p>0.05), and in the doxycycline-treated group it was 774±86 pg/ml (p>0.05).

As for SAA, in control group the level was 17±3 μg/ml, in the AVE 0991-treated group it was 8±1 μg/ml (p<0.05), in the nebivolol-treated group it was 14±2 μg/ml (p>0.05), and in the doxycycline-treated group it was 16±2 μg/ml (p>0.05) (Figures 1–4).

DISCUSSION

Angiotensin II (Ang II) is involved in physiology and pathology of the cardiovascular system [22]. Recent evidence shows that the renin-angiotensin system is a crucial player in the atherosclerotic processes [23]. It was also proved that Ang II promotes atherogenesis. On the other hand, angiotensin-(1-7) [Ang-(1-7)] opposes Ang II action [24]. Therefore, we would like to find out whether angiotensin-(1-7) receptor agonist: AVE 0991, could ameliorate atherosclerosis progression in an experimental model of atherosclerosis: apolipoprotein E (apoE) – knockout mice [25].

The renin-angiotensin system (RAS) is a highly complicated hormonal system controlling the cardiovascular system, kidney and adrenal glands, and is thus crucial for hydroelectrolyte balance and blood pressure regulation [22].

Ang II is the best described peptide of RAS. Ang II increases activity of sympathetic nervous system, acts as a vasoconstrictor, and increases aldosterone release and sodium retention [26]. Additionally, Ang II stimulates free radical production, plasminogen activator inhibitor-1 (PAI-1) release, and expression of tissue factor (TF) and adhesion molecules (VCAM-1). Moreover, in blood vessels it stimulates smooth muscle cell proliferation and leukocyte adhesion. Importantly, Ang II inhibits nitric oxide synthase (NOS), thus diminishing all beneficial effects of nitric oxide (NO).

Ang-(1-7) is an active peptide of RAS. It counteracts vasoconstriction by releasing nitric oxide and prostacyclin. Moreover, it opposes Ang II mitogenic, arrhythmogenic and procoagulant activities [27]. Enhancing natriuresis and diuresis, it inhibits water and sodium retention caused by Ang II. Recently it has been shown that vasodilatative and diuretic activities of Ang-(1-7) are mediated via Mas, G-coupled protein receptor [28]. Furthermore, some activities of Ang-(1-7) are blocked by AT_1 and AT_2 receptors antagonists [29]. On the other hand, Ang-(1-7), independent of Mas-receptor, increases bradykinin activity and antagonizes hypertrophic action of Ang II [30]. In 2002 non-peptide antagonist of Ang-(1-7) receptor: AVE 0991 has been described [31–33].

Ang II has several potential mechanisms that may increase the atherogenic process [34]. First, Ang II may indirectly influence the atherogenic process via hemodynamic effects resulting

from increased arterial blood pressure. Marked increases in arterial blood pressure have been demonstrated to increase the severity of experimental atherosclerosis. Second, Ang II has been demonstrated to exert several direct effects relevant to the development of atherosclerosis, including stimulation of monocyte recruitment, activation of macrophages, and enhanced oxidative stress, all of which have been linked to an increase in the atherogenesis process. These effects of Ang II occur independent of elevations in arterial blood pressure. In our experiment we have shown that AVE 0991 inhibits atherogenesis in a mouse model of atherosclerosis. This phenomenon is in agreement with general "anti-Ang II" action of Ang-(1-7). Since Ang II is a potent proatherogenic agent, its functional antagonist was able to diminish all markers of inflammation to a statistically significant degree.

The endothelium plays a crucial role in vessel wall homeostasis and its inflammatory and proliferative phenotype influences the progression of atherosclerosis [34]. Nebivolol, a novel third generation β_1-selective antagonist has been shown to increase bioavailability of endothelium-derived nitric oxide (NO) and to attenuate inflammatory activation of endothelial cells [35]. Here, using an atherosclerosis model of apoE-*knockout* mice, we confirmed the anti-atherogenic action of nebivolol. Although the details of endothelial action remain unclear, it seems that nebivolol augments vascular nitric oxide release *via* endothelial β_2- or β_3-adrenergic receptors [36,37]. Furthermore, it was shown that nebivolol prevents vascular nitric oxide synthase (NOS) III uncoupling in experimental hyperlipidemia and inhibits NADPH oxidase activity in endothelial and inflammatory cells [37,38]. Recently, nebivolol appeared to be a potent antioxidant and has been shown to reduce expression of inflammatory adhesion molecules (ICAM-1, E-selectin) and cytokines (TNF-α, IL-6), as well as prothrombotic factors (PAI-1) on endothelial and smooth muscle cells [39]. Our preliminary data show that inhibition of atherogenesis by nebivolol in apoE knockout mice is associated with its tendency to decrease of plasma sICAM-1 and VCAM-1 levels (unpublished data). Furthermore, Baumhakel et al. reported that nebivolol, but not metoprolol, improved endothelial function of the corpus cavernosum in apoE-*knockout* mice [40]. Interestingly, the third generation of β-adrenoreceptor antagonists with ancillary vasodilator properties (nebivolol and carvedilol) possesses superior clinical efficacy as compared to the classical β-blockers [41,42]. This seems to be related not to their β-blocking properties, but to their ability to reverse endothelial dysfunction. Indeed, nebivolol, but not atenolol, reversed endothelial dysfunction in patients with heart failure and hypertension [43,44]. Our data showed an anti-atherogenic action of nebivolol. However, even though there was a tendency to diminish the level of inflammatory markers, it did not reach statistical significance.

Extracellular matrix (ECM) degradation is tightly regulated within the normal vessel wall through a balance between proteinases and their endogenous inhibitors. However, within the atherosclerotic plaque the balance may become shifted towards matrix degradation, since accumulating macrophages and phenotypically altered smooth muscle cells secrete a plethora of proteinases, including matrix metalloproteinases (MMPs) [45].

Although undetectable in normal arteries, MMP-1 expression has been localized to the fibrous cap and the shoulder regions of carotid atherosclerotic lesions [46]. In the latter tissue, the cellular sources of MMP-1 are mainly represented by macrophages, smooth muscle cell SMCs, and endothelial cells. Morphological analysis of the plaques have, in addition, revealed higher MMP-1 transcript levels in carotid lesions with a large lipid core and thin fibrous cap as compared with fibrous lesions with thick fibrous caps [47]. The latter findings suggest an increased MMP-1 expression associated with plaque vulnerability, which has also been supported by the MMP-1 messenger RNA (mRNA) levels detected in carotid lesions derived from patients with recent ischemic manifestations. Finally, a study of carotid lesions derived from patients undergoing repeated vascular intervention has shown that an increased MMP-1 expression correlated with the more foam cell-dominated late lesions compared with early restenotic lesions, which were characterized by increased SMC content [48]. Taken together, these studies support a role of MMP-1 derived from inflammatory cells in ECM degradation associated with plaque rupture [49].

Exogenous synthetic inhibitors generally contain a chelating group that binds the catalytic zinc atom in the enzyme active site. Doxycycline, already at sub-antimicrobial doses, inhibits MMPs activity, and has been used in various experimental setups for this purpose [50]. It is used clinically for the treatment of periodontal disease and is the only MMP inhibitor that is widely available clinically [51,52]. MMPs have been also considered as putative therapeutic targets in the prevention of atherogenesis.

In experiments made by Madan et al., there was a positive effect of doxycycline on atherogenesis in a special apoE heterozygote murine model infected with *Porphyromonas gingivalis* [53]. Therefore, our data, provided on apoE-*knockout* mice, broaden the current knowledge about the positive effect of doxycycline in sub-antimicrobial doses on atherogenesis. Here, as in the case of nebivolol, there was a tendency to diminish the level of inflammatory markers, which, however, did not reach statistical significance.

CONCLUSIONS

From the 3 checked substances – AVE 0991, nebivolol and doxycycline – that were able to inhibit atherosclerosis (an inflammatory disease), only AVE 0991 was at the same time able to diminish the rise of inflammatory markers. Therefore, drug manipulations in the renin- angiotensin-aldosterone axis seem to be the most potent and promising in the future treatment of atherogenesis.

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