Pharmacological Reports 71 (2019) 551–555

Contents lists available at ScienceDirect

Pharmacological Reports

journal homepage: www.elsevier.com/locate/pharep

Short communication

Anti-atherosclerotic action of GW9508 – Free fatty acid receptors activator – In apoE-knockout mice

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ARTICLE INFO

Article history:
Received 16 January 2019
Received in revised form 18 February 2019
Accepted 20 February 2019
Available online 21 February 2019

Keywords:
GW9508
Free fatty acid receptors
Atherosclerosis
apoE-knockout mice
Macrophages

ABSTRACT

Background: In the past two decades, enhanced understanding of the biology of G-protein-coupled receptors (GPRs) has led to the identification of several such receptors as novel targets for free fatty acids (FFAs). Two GPRs, FFAR1 and FFAR4, have received special attention in the context of chronic inflammatory diseases, thanks to their anti-inflammatory activities.
Methods: The present study investigates the influence of prolonged treatment with GW9508 – agonist of FFAR1 and FFAR4 – on the development of atherosclerosis plaque in apoE-knockout mice, using morphometric and molecular methods.
Results: GW9508 administration has led to the reduction of atherosclerotic plaque size in an apoE-knockout mice model. Moreover, a FFAR1/FFAR4 agonist reduced the content of macrophages by almost 20%, attributed by immunohistochemical phenotyping to the pro-inflammatory M1-like activation state macrophages.
Conclusions: Prolonged administration of GW9508 resulted in significant amelioration of atherogenesis, providing evidence that the strategy based on macrophage phenotype switching toward an M2-like activation state *via* stimulation of FFAR1/FFAR4 receptors holds promise for a new approach to the prevention or treatment of atherosclerosis.

Introduction

In the past two decades, enhanced understanding of the biology of G-protein-coupled receptors (GPR) has led to the identification of several such receptors as novel targets for free fatty acids (FFAs) [1]. The FFAs are the ligands for GPR40 (FFAR1), GPR43 (FFAR2), GPR41 (FFAR3), GPR120 (FFAR4), GPR119, and GPR84; the binding of FFAs to receptors varies depending on the number of carbons and unsaturated bonds in a molecular structure. FFAR1 and FFAR4, both coupled with a G-protein α-subunit of the Gq family, recognize medium- and long-chained FFAs, respectively. FFAR1 and FFAR4 have received special attention in the context of chronic metabolic and cardiovascular diseases, thanks to their endocrine and anti-inflammatory activities.

Importantly, *in vitro* data suggest that, in different cell types (*e.g.*, adipocytes, hepatocytes, muscles, epithelial cells, and macrophages), stimulation of FFAR1 and FFAR4 by ω-3 and ω-9 PUFAs exerts potent anti-inflammatory action. Oh et al., in an elegant paper, demonstrated that *in vitro* stimulation of macrophage FFAR4 by ω-3 PUFAs or a synthetic agonist (GW9508) caused a broad anti-inflammatory response by interfering with the LPS- and TNF-α-induced signaling cascades [2]. Moreover, upon activation of FFAR4, the expression of several inflammatory genes and markers in macrophages was significantly altered, pointing to a shift from a pro-inflammatory M1-like activation state toward an anti-inflammatory, pro-resolution, M2-like phenotype [2]. Yan et al.

Abbreviations: AMPK, 5'AMP-activated protein kinase; apoE$^{-/-}$, apoE-knockout mine; DAPI, 4',6-diamidino-2-phenylindole; DMSO, dimethyl sulfoxide; DTT, dithiothreitol; EPA, eicosapentaeonic acid; FFA, free fatty acid; FFAR, free fatty acid receptor; GADPH, glyceraldehyde 3-phosphate dehydrogenase; GPR, G-protein-coupled receptor; HDL, high density lipoproteins; IL-1β, interleukin 1 beta; iNOS, inducible nitric oxide synthase; LDL, low density lipoproteins; LPS, lipopolysaccharide; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; OCT, optimal cutting temperature; PPARγ, peroxisome proliferation activator receptor gamma; PUFA, polyunsaturated fatty acid; RT-PCR, reverse transcription polymerase chain reaction; SMA, smooth muscle actin; TC, total cholesterol; TG, triglyceride; TLR, toll-like receptor.

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https://doi.org/10.1016/j.pharep.2019.02.014
1734-1140/

found that stimulation of macrophage FFAR1 and FFAR4 with ω-3 PUFAs inhibited the NLRP3 inflammasome and its subsequent IL-1β secretion [3]. Finally, both FFAR1 and FFAR4 have been linked with the downstream activation of 5′AMP-activated protein kinase (AMPK), which orchestrates a metabolic reprogramming of macrophages between M1- and M2-like activation states [4].

These observations are of particular interest in the context of recent conceptual changes regarding the mechanisms by which different types of leukocytes contribute to atherogenesis. Over the last decade, the paradigm of the predominant circulatory origin of lesional macrophages has been challenged by the characterization of several effects that take place within the plaque, including macrophage proliferation [5], vascular smooth muscle cell (VSMC) transdifferentiation to macrophages [6], and macrophage polarization [7].

In the present study, we investigate the influence of prolonged treatment with GW9508 – agonist of FFAR1 and FFAR4 – on the development of atherosclerosis in apoE-knockout (apoE-/-) mice, using morphometric and molecular methods. We hypothesize that FFAR1/FFAR4 activation may counteract the pro-inflammatory activation of macrophages and therefore possibly facilitate pro-resolution polarization of these cells in a lesional milieu.

Materials and methods

Animal experiments

All animal procedures were approved by the Jagiellonian University Ethical Committee on Animal Experiments. Female apoE-knockout mice on the C57BL/6 J background were purchased from Taconic (Ejby, Denmark). The mice were housed in air-conditioned rooms (22.5 ± 0.5 °C, 50 ± 5% humidity) with 12-h dark/12-h light cycles, with access to diet and water *ad libitum*. Eight-week-old mice were put on a chow diet made by Morawski (Kcynia, Poland). Two groups of animals were studied: a control group (apoE$^{-/-}$ mice with *ip* injections of 100 μl of DMSO three times a week, n = 15) and GW9508-treated mice (n = 8). In the latter group, 0.5 mg of GW9508 dissolved in DMSO was injected *ip* to mice three times a week (25 mg/kg of body weight). After four months, the mice were injected with 1000 IU of fraxiparine (Sanofi-Synthelabo, France) into the peritoneum and sacrificed in a carbon dioxide chamber. Next, the blood was collected, while hearts and aortas were dissected. One mouse in the control group and four animals in the GW9508 group did not complete the study. Postmortem inspections of these animals revealed signs of adhesions and inflammatory changes in the abdomen area, which we attribute to the multiple injections performed during the study.

Analysis of atherosclerotic plaque

The hearts with the ascending aorta were embedded in OCT compound (CellPath, Newtown, UK), snap frozen and sectioned (10 μm thickness) for histological and immunohistochemical analysis, according to the standardized cross-section protocol, as previously described [8]. To evaluate the lesion area, nine sections per animal were stained with Oil Red O (Sigma-Aldrich, St. Louis, MO, USA). Immunohistochemistry for total macrophages and smooth muscles was performed with antibodies against CD68 (dilution 1:800; Serotec, Kidlington, UK) and smooth muscle α-actin (SMA) (dilution 1:800; Sigma-Aldrich). Macrophage polarization was assessed with antibodies against F4/80 (dilution 1:100), nitric oxide synthase 2 (iNOS) (dilution 1:200), arginase 1 (dilution 1:100), and 4′,6-diamidino-2-phenylindole (DAPI), for total macrophages, M1-like, M2-like, and cell nuclei, respectively. All section images were captured using an Olympus Camedia DP71 digital camera and analyzed using LSM Image Browser software (Zeiss, Jena, Germany). Additional validation of staining protocol and fluorescent signal co-localization was performed by confocal microscopy.

Morphometric estimation of the plaque macrophage polarization was done by immunohistochemical detection and quantitation of the respective cell phenotype. Cells with detected co-localization of three fluorescent signals from DAPI (nuclei), F4/80 (total macrophages), and iNOS/arginase-1 antigens for M1/M2 phenotype, respectively, were counted and divided by the number of total macrophages visualized. Only clear orange signals (being the result of the superposition of red and green channels) surrounding blue nuclei were considered as positive matches for the respective macrophage subtype. Altogether, four hearts from each group were subjected to analysis, with at least two immunohistochemical images per phenotype per biological replicate examined. Collectively, 34 merged images encompassing visualization of both phenotypes were analyzed independently by three examiners and averaged for final macrophage subtype quantitation.

Biochemical methods

The blood was collected from the right ventricle and centrifuged for 10 min, 1000 *g* at 4 °C. Plasma was harvested and stored in −80 °C until assayed. The levels of total cholesterol, triglycerides, and low- and high-density lipoproteins (LDL and HDL) were measured using an enzymatic method on a Cobas 8000 analyzer (Roche Diagnostics, Indianapolis, IN, USA).

Real time RT-PCR

Total RNA was isolated from the homogenized mouse aortas using the RNeasy Fibrous Tissue Mini Kit (Qiagen, USA), according to the manufacturer's instructions. The RNA concentration of each sample was measured at a wavelength of 260 nm (A260) in an EPOCH microplate spectrophotometer (BioTek Instruments, Inc., Winooski, VT, USA). The purity of extracted total RNA was determined by the A260/A280 ratio. cDNA was synthesized by the reverse transcription of 1000 ng of total RNA from each sample, using a High-Capacity Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). The cDNA was diluted ten-fold prior to PCR amplification.

Real time quantitative PCR, using GoTaq® qPCR Master Mix (Promega, Madison, WI, USA), was carried out on the Viia7 Real Time PCR System (Applied Biosystems). Primers for GAPDH, CD68, iNOS, IL-1β, TNF, CD206, IL-10, and MGL-1 were purchased from RealTimePrimers.com (Elkins Park, PA, USA). Analysis of the data was performed by the $2^{-\Delta\Delta Ct}$ method using Data Assist v3.01 software (Applied Biosystems), and GAPDH expression was used as the internal control.

Statistics

The equality of variances was assessed by the Fischer test, while the normality of the data was checked by the Shapiro–Wilk test. A *t*-test was used to assign the statistical significance of the data in GraphPad Prism 8.0.1 (GraphPad Software, San Diego, CA, USA). A value of $p < 0.05$ was considered as statistically significant.

Results

Effects of GW9508 on atherosclerosis

Treatment with GW9508 did not influence the plasma lipid profile in apoE-/- mice. The levels of total cholesterol, HDLs, LDLs, and TGs in blood were slightly increased; however, measured differences did not reach statistical significance (Table 1).

Table 1
Plasma lipids level in control and GW9508-treated groups.

	TC (mmol/L)	HDL (mmol/L)	LDL (mmol/L)	TG (mmol/L)
Control (n = 14)	10.33 ± 1.73	3.90 ± 2.15	7.30 ± 1.44	0.78 ± 0.09
GW9508 (n = 4)	11.13 ± 3.05	4.27 ± 2.35	9.00 ± 2.65	1.11 ± 0.39

Data presented as mean ± SD. TC - total cholesterol; TG - triglyceride; HDL - high-density lipoproteins; LDL - low-density lipoproteins.

As evidenced by the cross-section method, treatment with GW9508 reduced the area of atherosclerotic lesions in apoE$^{-/-}$ mice by approximately 35% (49,364 ± 16,959 μm^2 and 75,617 ± 13,969 μm^2 for the FFARs agonist group and control apoE-/- mice, respectively) (Fig. 1). Moreover, GW9508 treatment significantly reduced the macrophage-like cell content in the atherosclerotic lesions by 20%, as evidenced by CD68 staining (Fig. 1), and simultaneously but not statistically significantly increased the number of smooth muscle cells in the fibromuscular cap by 44%, as evidenced by α-SMA staining (Fig. 1).

In-plaque macrophage phenotyping

Since the total content of CD68-positive cells was reduced by GW9508 treatment, we investigated whether the quantitative changes are accompanied by qualitative differences within the plaque macrophage pool. Indeed, GW9508 modified the phenotype of macrophages in the atherosclerotic lesions in apoE$^{-/-}$ mice. In our study, the pro-inflammatory M1-like activation state population, detected by iNOS-positive staining, was reduced by approximately 35% in the GW9508 group compared to the control apoE$^{-/-}$ mice, while the number of pro-resolution M2-like phenotype cells, visualized by arginase 1-positive signals, did not change upon FFAR receptor activation (Fig. 2). Thus, the relative ratio of pro-resolution M2-like cells in the plaque macrophage pool increased. Gene expression analysis confirmed the reduction of total macrophage content in the aortas of GW9508-treated apoE$^{-/-}$ mice. Moreover, in the aortas of GW9508-treated animals, the downregulation of iNOS transcript was more pronounced than CD206 mRNA repression (56% and 50% reduction, respectively), which supported morphometric phenotyping.

Discussion

Recent advances in atherosclerosis research in the field of mechanistic cellular pathobiology indicate the need to revise the classical paradigms of the cellular mechanisms of atherogenesis. For instance, the commonly accepted notion that lesional macrophages and media-derived VSMC exhibit contrary characteristics in terms of plaque stability needs to be reevaluated, in view of discoveries from VSMC tracing studies [6]. New developments have evidenced a high degree of plasticity of macrophages and VSMC within the plaque, showing that macrophages respond to changes in their extra- and intracellular environment by giving rise to specific phenotypes [9], as well as that macrophage-like cells can differentiate from smooth muscle cells and, intriguingly, *vice versa* [6,10].

The main finding of our study is that activation of FFARs by GW9508 led to the reduction of atherosclerotic plaque size in an apoE-knockout mice model. Importantly, the FFAR1/FFAR4 agonist led to qualitative modifications in cellular lesion composition, reducing the content of macrophages by almost 20%. Detailed immunohistochemical evaluation of plaque composition,

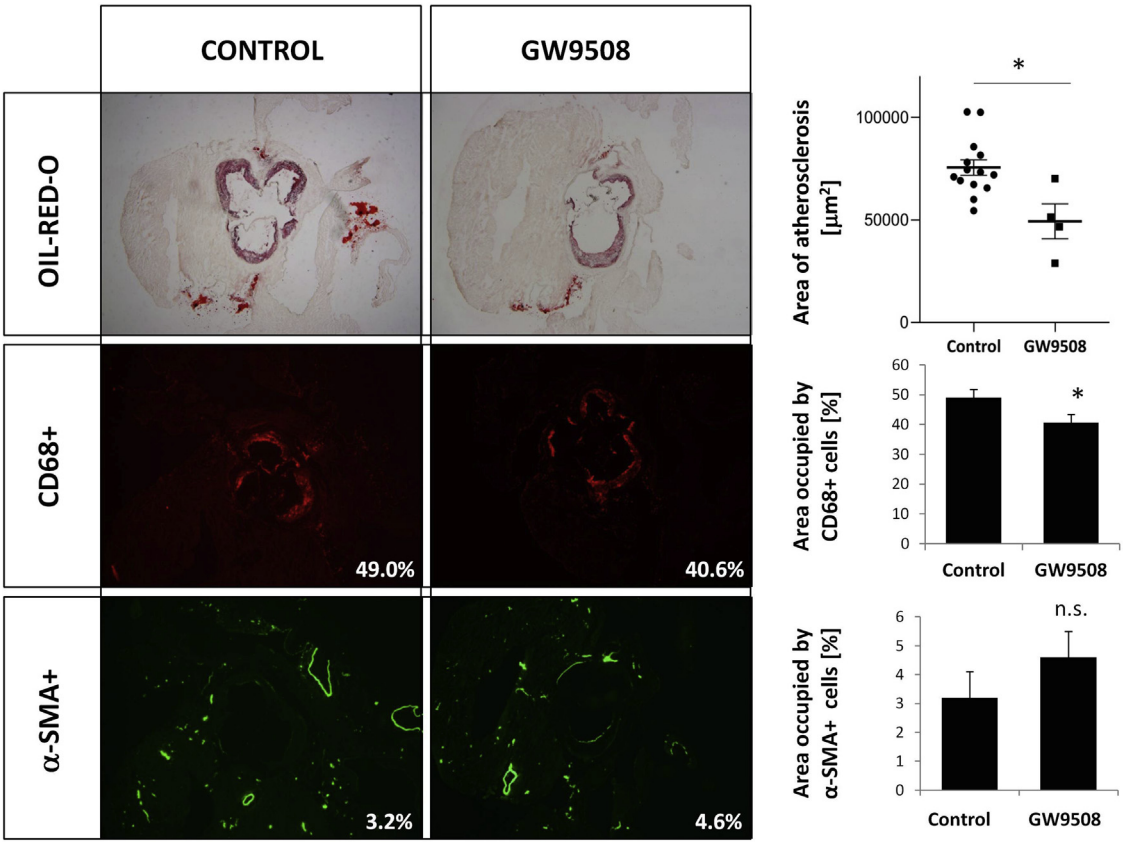

Fig. 1. Effects of GW9508 on atherosclerosis progression. Representative Oil Red O, CD68-positive (red) and α-SMA-positive (green) stained atherosclerotic lesions measured in aortic roots by the cross-section method in control group (n = 14) and GW9508-treated group (n = 4). Graphs represent mean ± SEM values, (*$p < 0.05$).

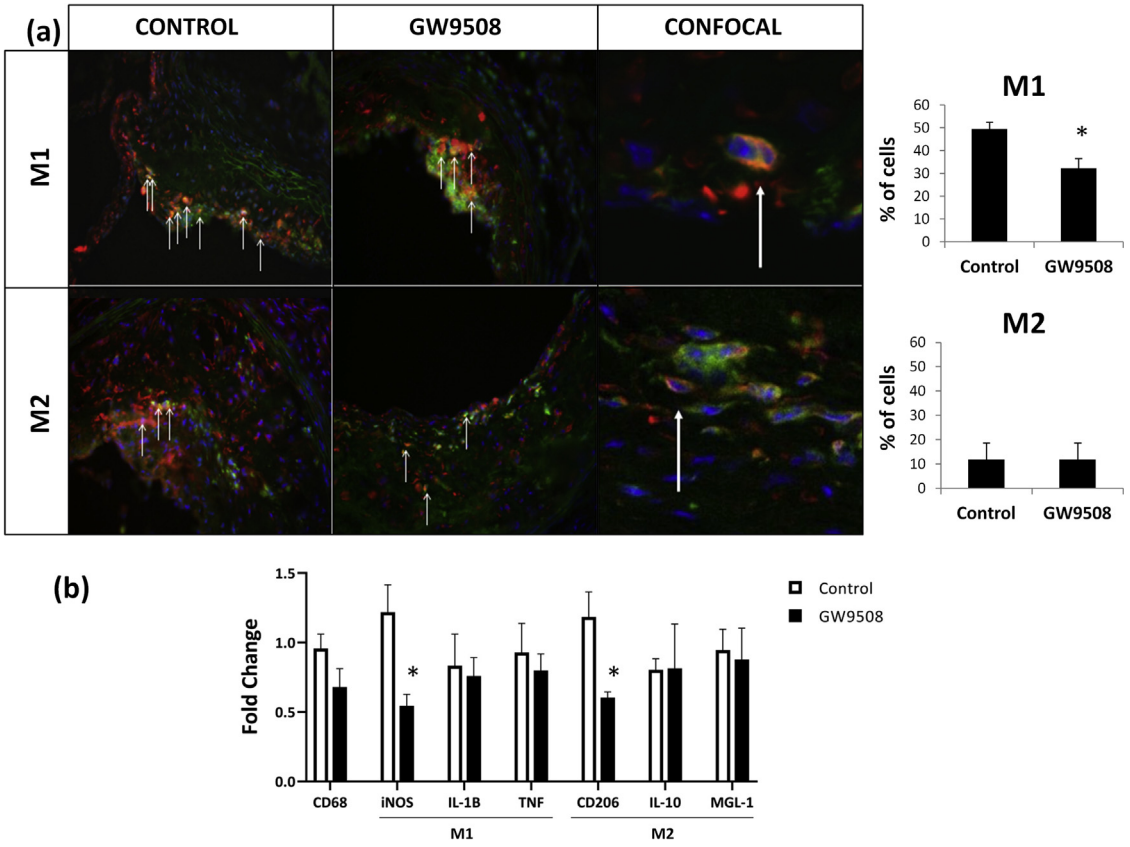

Fig. 2. Macrophage phenotyping in atherosclerotic plaque. Representative micrographs showing immunohistochemical staining of aortic roots from control (n = 4) and GW9508-treated (n = 4) apoE-knockout mice. F4/80 (green), nitric oxide synthase 2 (iNOS)/arginase 1 (red) and 4′,6-diamidino-2-phenylindole (DAPI) (blue) co-localization, additionally evidenced by confocal microscopy, confirming the respective macrophage phenotype (white arrows). Adjacent graphs represent mean ± SEM values, (* $p < 0.05$) (a). Validation of macrophages morphometry by RT-PCR analysis (n=5 for control and n=3 for GW-treated mice) confirmed a trend toward reduction of total

supported by molecular measurements, indicated that pro-inflammatory M1-like activation state macrophages (F4/80-iNOS positive cells) were significantly reduced in GW9508-treated apoE$^{-/-}$ mice. Our study is the first to describe the effects of GW9508 administration in an apoE-knockout experimental model.

The discrepancy between the effect of FFAR1/FFAR4 agonist on macrophages at different activation states assessed in our study can be explained by the expression level of FFAR receptors in these populations. The FFAR4 induction has been attributed specifically to the M1-like phenotype, while the FFAR1 expression level on macrophages is low [2]. Moreover, whereas FFAR4 has been linked to the reduction of pro-inflammatory activation of macrophages, the anti-inflammatory effects of FFAR1 remain obscure [2]. It should be noted that our results are in line with the study of Matsumoto et al., which described the reduction of aortic lipid deposition and plaque macrophage content, accompanied by the elevation of the plaque collagen deposits in apoE-knockout model administered eicosapentaeonic acid (EPA), which is a known activator of FFAR4 [11].

Although challenged by some recent data [12], the beneficial cardiovascular effects of polyunsaturated fatty acids (PUFAs) are widely accepted. There is a growing body of evidence from both animal and human studies that PUFAs hold moderate anti-atherogenic potential [13]. Several general mechanisms of the action of PUFAs have been proposed to date, including stabilization of the vulnerable plaque, reduction of platelet aggregation, the lowering of plasma TG levels, or decreased infiltration of immune cells into the plaque [14]. On the molecular level, PUFAs have been proposed to inhibit toll-like receptors, activate peroxisome proliferation activator receptor gamma (PPARγ), or compete with arachidonic acid as a substrate for the major inflammatory enzymes of the eicosanoid pathway (cyclooxygenase 2, lipoxygenases), resulting in the synthesis of pro-resolving mediators (resolvins, protectins, maresins) [14]. For instance, it has been demonstrated that the synthesis of chemoattractant leukotriene B_4 can be reduced by PUFAs, and such action may lead to a decrease in the recruitment of leukocytes to the lesions [14]. Moreover, PUFAs could reduce the expression of chemokine receptors on leukocytes, which further attenuates plaque infiltration [15]. It is noteworthy that the key hallmark of inflammation – activation of the NF-κB transcription factor pathway – is perturbed in the presence of PUFAs, and in macrophages this action has been directly attributed to FFAR4 activation [2]. Indeed, PUFAs are responsible for several important downstream, anti-atherogenic consequences of NF-κB inhibition, including a decrease in the production of cytokines and the expression of adhesion molecules on the surface of different cell types contributing to atherosclerotic plaque formation, including endothelial cells, monocytes, and macrophages [16,17].

All of the abovementioned effects of PUFAs signal their involvement in a cellular phenotype switching phenomenon, which is increasingly recognized as a potential target for future pharmacotherapies of atherosclerosis, especially for individuals who do not respond to the classical lipid-lowering strategies [18]. Indeed, if atherosclerosis is considered as a low-grade, unresolved inflammation, the phenotype switching approach, favoring a macrophage M2-like activation state, with increased capability

for the safe removal of chronically activated cells, could hold promise as an improved atherosclerosis treatment. Our study strengthens these expectations, providing evidence that stimulation of free fatty acids receptors by the administration of their synthetic agonist GW9508 results in the attenuation of atherosclerosis in an apoE$^{-/-}$ mice model by targeting pro-inflammatory M1-like macrophages and shifting the balance within plaques toward cells of M2-like phenotype.

Clearly, our study has a major limitation in that several of the GW9508-treated apoE$^{-/-}$ mice did not reach the end of the experimental protocol. Thus, when taking measurements, we could detect only trends in the changes of several parameters, not reaching statistical significance, thus making our mechanistic conclusions preliminary. This eventuality was somewhat surprising, since the GW9508 compound has been previously used in mice, usually administered intraperitoneally or orally, even at four-fold higher doses than in our setting [19,20]. The major discrepancy, apart from different mouse models investigated, was the duration of the experiments, ranging from seven to 30 days in the abovementioned studies, whereas our study continued for 16 weeks to ensure the development of sufficient atherosclerotic changes on a chow diet. Therefore, further investigation of the anti-atherosclerotic effect of GW9508, as well as exploration of the specific mechanisms elicited by FFAR1/FFAR4 activation in apoE$^{-/-}$ mice, is undoubtedly required. Different formulation of the GW9508 solution and other routes of drug administration should be considered in long-term experiments carried out on the apoE$^{-/-}$ mice model.

Conclusions

Prolonged administration of GW9508, synthetic FFAR1, and FFAR2 agonist resulted in a significant amelioration of the formation of atherosclerotic plaques and a decrease in the macrophage content in apoE-knockout mice. The anti-atherosclerotic action of GW9508 was associated with a reduction in pro-inflammatory M1-like activation state macrophages and, in turn, with a relative increase in the M2/M1 cell ratio in the total macrophage pool.

Author contributions

Conceptualization: M. Suski, R. Korbut and R. Olszanecki; Formal analysis: M. Suski, A. Kiepura, A. Wiśniewska, K. Kus, K. Stachyra and A. Stachowicz; Investigation: A. Kiepura, A. Wiśniewska, K. Kus and A. Skałkowska; Methodology: A. Kiepura, A. Wiśniewska and M. Gajda; Supervision: R. Korbut and R. Olszanecki; Writing – original draft: M. Suski and R. Olszanecki.

Funding

This research was funded by National Science Centre, grant number 2015/17/D/NZ2/01972.

Conflicts of interest

The authors declare no conflict of interest.

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