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Glucagon-like peptide-1 (GLP-1) and its split products GLP-1(9-37) and GLP-1(28-37) stabilize atherosclerotic lesions in apoe$^{-/-}$ mice

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ABSTRACT

Backround: Glucagon-like peptide-1 (GLP-1) based therapies are new treatment options for patients with type 2 diabetes. Recent reports suggest vasoprotective actions of GLP-1. Similar beneficial effects might be reached by GLP-1(9-37) and the c-terminal GLP-1 split product (28-37) although both peptides do not activate the GLP-1 receptor. We therefore investigated the actions of GLP-1(7-37), GLP-1(9-37) as well as GLP-1(28-37) on vascular lesion formation in a mouse model of atherosclerosis.

Methods and results: GLP-1(7-37), GLP-1(9-37) and GLP-1(28-37) and LacZ (control) were overexpressed for a period of 12 weeks in apoe$^{-/-}$ mice on high-fat diet ($n = 10$/group) using an adeno-associated viral vector system. Neither of the constructs changed overall lesion size. However, GLP-1(7-37), GLP-1(9-37) and GLP-1(28-37) significantly reduced plaque macrophage infiltration (GLP-1(7-37): 40.6%, GLP-1(9-37): 47.0%, GLP-1(28-37): 40.1% decrease, $p < 0.05$) and plaque MMP-9 expression (GLP-1(7-37): 41.6%, GLP-1(9-37): 50.2%, GLP-1(28-37): 44.0% decrease, $p < 0.05$) compared to LacZ in the aortic arch. Moreover, all GLP-1 constructs increased plaque collagen content (GLP-1(7-37): 49.3%, GLP-1(9-37): 86.0%, GLP-1(28-37): 81.9% increase, $p < 0.05$) and increased fibrous cap thickness (GLP-1(7-37): 188.0%, GLP-1(9-37): 179.9% GLP-1(28-37): 111.0% increase, $p < 0.05$).
These effects of GLP-1(7-37), GLP-1(9-37) and GLP-1(28-37) on plaque macrophage infiltration, MMP-9 expression and plaque collagen content were confirmed in the aortic root.

Conclusion: GLP-1(7-37), GLP-1(9-37) and GLP-1(28-37) reduce plaque inflammation and increase phenotypic characteristics of plaque stability in a murine model of atherosclerosis.
Future studies are needed to determine whether these effects translate into improved plaque stability and less cardiovascular events in high-risk patients with type 2 diabetes.

1. Introduction

Glucagon-like peptide-1 (GLP-1) based therapies are a new group of anti-diabetic drugs which improve glucose metabolism by increasing glucose dependent insulin secretion while suppressing glucagon release from pancreatic islet cells [1]. GLP-1 reduces gastric motility, suppresses appetite and has potent athero- and cardioprotective effects [2]. Bioactivity of GLP-1 is however limited to approximately 2 min due to a rapid DPP-IV dependent degradation via cleavage of the first two amino-acids of the peptide [1]. The thereby created GLP-1(9-37) does not stimulate the GLP-1 receptor but rather acts as a week antagonist [1]. Further degradation of GLP-1 is reached by neutral endopeptidase dependent cleavage at the c-terminus of the peptide creating the additional split products GLP-1(28-37) and GLP-1(32-37) [3].

GLP-1 based therapies take advantage of longer acting agonists of the GLP-1 receptor or inhibitors of dipeptidyl peptidase-IV (DPP-IV) which increase the bioavailability of endogenous GLP-1. As patients with type 2 diabetes are at a particular high risk for cardiovascular disease [4], protective cardiovascular effects of GLP-1 based therapies might hold great potential to limit cardiovascular risk in this population [2,5]. Beneficial cardiovascular effects of GLP-1 based therapies are currently emerging [2]. Specifically, we and others have demonstrated DPP-IV inhibition to reduce plaque inflammation and increase plaque collagen content in apoe$^{-/-}$ mice [6–9]. However, as DPP-IV has various substrates and thus degrades a large magnitude of peptides [10,11], it currently remains unknown to what extent these

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anti-inflammatory, plaque stabilizing effects can be attributed to GLP-1. Furthermore, not only GLP-1 but also its split products – whose creation get modulated by DPP-4 inhibition – might have cardiovascular actions [5]. The GLP-1 metabolite (9-37) which does not activate the GLP-1 receptor, was thereby found to have similar cardioprotective effects as GLP-1 in context of acute myocardial infarction [12,13]. These actions were preserved in the absence of the GLP-1 receptor suggesting the presence of an additional yet not identified receptor system [12]. Beneficial metabolic effects – including a reduction of body weight and improved insulin sensitivity have further been described for the c-terminal GLP-1 split product (28-37) [14,15]. Clear specification of cardiovascular actions of these split products under in vivo conditions are currently lacking. We therefore studied the vascular effects of GLP-1(7-37) and its split products GLP-1(9-37) and GLP-1(28-37) on plaque phenotype with a particular emphasis on features of plaque morphology and stability in apoe$^{-/-}$ mice as a mouse model of atherosclerosis.

2. Animal model and methods

2.1. Construction of recombinant adeno-associated viral constructs

Vectors carried transgene cassettes encoding β-galactosidase (LacZ) as control, GLP-1(7-37), GLP-1(9-37) or GLP-1(28-37) under control of a cytomegalovirus (CMV) promoter. The pseudotyping strategy was used to produce AAV vectors encapsidated in an AAV8 capsid (AAV2.8) as previously reported [16]. Cloning was inspected by sequencing and restriction digests. Vectors were purified by standard caesium sedimentation. Titers were determined via TaqMan RealTime polymerase chain reaction (PCR) as described elsewhere [17].

2.2. Animals

Forty C57BL/6 apoE$^{-/-}$ mice were purchased from Taconic, USA, at the age of 6 weeks and kept two weeks on normal chow in order to adjust to the new environment. Animals were housed in the animal facility of the University Hospital of the RWTH Aachen in accordance with German guidelines for care and use of laboratory animals. At the age of 8 weeks mice were matched to experimental groups under consideration of total serum cholesterol and triglycerides. Ten mice per group received respective AAV vectors via tail vein injection. Mice were switched to a western diet (ssniff, Germany) 4 weeks after vector injection on which they remained for a total of 12 weeks. One mouse in the GLP-1(7-37) and one mouse in the GLP-1(9-37) group died during the experimental period leaving 9-10 mice per group for final analysis.

Body weight was monitored weekly. An oral glucose tolerance test (OGTT) was performed after 10 weeks on western diet following a 6 h fasting period. 2 g glucose per kg body weight were administrated by oral gavage. Blood glucose levels were measured with a glucometer (Contour, Bayer, Germany) at 0, 30, 60, 90, 120 min after the glucose administration. After 12 weeks on western diet mice were anesthetized with Isoflurane (Abott, Germany) and euthanized by cervical dislocation.

2.3. Serum lipid measurement

Quantitative determination of cholesterol and triglycerides in serum was performed by enzymatic CHOD-PAP method using diagnostic reagents for photometric systems (DiaSys Diagnostic, Germany) and photometer (TECAN, Switzerland) at 546 nm as described elsewhere [18].

2.4. Determination of GLP-1 levels

Blood was collected after a 6 h fasting period and treated with a DPP-4 inhibitor (Calbiohem).

For determination of GLP-1(7-37) and (9-37) serum levels, specific capture antibody (Dianova, Germany) against the C-terminus of the GLP-1 molecule was coated directly onto the microtiter well bottom to bind GLP-1 from serum. A biotinylated secondary antibody against mid-molecular epitope (12–18) (Dianova, Germany) was used as detection antibody. Excitation was measured at 450 nm in a plate reader (Tecan, Swizerland).

For expression of GLP-1(28-37) standard western blotting procedure was performed with pooled plasma samples using specific antibody against non-amidated C-terminus (Dianova, Germany). Synthetic GLP-1(7-37) was used as a control. Western blot images were analysed using densitometry. Serum levels of GLP-1(28-37) were calculated by relative changes as compared to GLP-1 serum concentration in the GLP-1(7-37) group.

2.5. Organ collection and processing

Aorta and heart were carefully perfused with PBS to remove the blood. The cardiac apex was cut off and the aortic arch was separated from aorta distal of the left subclavian artery, embedded in freezing medium and stored by −80 °C.

2.6. Atherosclerotic lesion assessment

To visualize plaque extension in the descending aorta, Oil-red-O staining was performed as previously described [6]. Briefly, fixed and pinned aortas were rehydrated in PBS, washed in 100% propylene glycol and incubated for 20 min with Oil-red-O solution, subsequently counterstained in haematoxyllin. Oil-red-O staining of aortic arch and aortic root sections was used to determine the lipid content of the plaque. Haemotoxillin staining was used to visualize the plaque and to assess the plaque size in the aortic root and the aortic arch.

Relative lesion size of descending aorta and absolute size in aortic root and aortic arch was determined using an image processing software (Image Pro-Plus, Media Cybernatics, USA). Extent of atherosclerotic plaques is expressed in the aortic root and arch as μm^2 and in the descending aorta (en face) normalized to LacZ relative to surface area.

2.7. Immunohistochemistry

To visualize plaque macrophage content, acetone-fixed aortic root and aortic arch sections were stained with anti-mouse MOMA-2 antibody (AbD Serotecec, UK) using a biotinylated secondary antibody and haematoxylin and eosin for nuclear staining. Positive staining areas were selected by an investigator blinded to the treatment and analysed by an image processing software (Image Pro-Plus, Media Cybernatics, USA).

To investigate the expression of MMP-9 in the atherosclerotic plaques, an immunostaining was performed using a specific antibody against MMP-9 (Abcam, UK) as reported before [6]. To quantify the plaque collagen content sections were fixed with 4% paraformaldehyde and stained with 0.1% Sirius red solution as described elsewhere [6]. For the evaluation pictures of all stained sections were taken under a light microscope (Leica, Germany). Extent of macrophage accumulation (MOMA-2), MMP-9 expression and collagen content were expressed as percent of the lesion area.