ARTICLE

Sitagliptin reduces plaque macrophage content and stabilises arteriosclerotic lesions in *Apoe*$^{-/-}$ mice

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Abstract

Aims/hypothesis Inhibitors of dipeptidyl peptidase-IV (DPP-IV), such as sitagliptin, increase glucagon-like peptide-1 (GLP-1) concentrations and are current treatment options for patients with type 2 diabetes mellitus. As patients with diabetes exhibit a high risk of developing severe atherosclerosis, we investigated the effect of sitagliptin on atherogenesis in *Apoe*$^{-/-}$ mice.

Methods *Apoe*$^{-/-}$ mice were fed a high-fat diet and treated with either sitagliptin or placebo for 12 weeks. Plaque size and plaque composition were analysed using Oil Red O staining and immunohistochemistry. Furthermore, in vitro experiments with the modified Boyden chamber and with gelatine zymography were performed to analyse the effects of GLP-1 on isolated human monocyte migration and metalloproteinase-9 (MMP-9) release.

Results Treatment of *Apoe*$^{-/-}$ mice with sitagliptin significantly reduced plaque macrophage infiltration (the aortic root and aortic arch both showing a 67% decrease; $p<0.05$) and plaque MMP-9 levels (aortic root showing a 69% and aortic arch a 58% reduction; both $p<0.01$) compared with controls. Moreover, sitagliptin significantly increased plaque collagen content more than twofold (aortic root showing an increase of 58% and aortic arch an increase of 73%; both $p<0.05$) compared with controls but did not change overall lesion size ($8.1\pm3.5\%$ vs $5.1\pm2.5\%$ for sitagliptin vs controls; $p=$NS). In vitro, pretreatment of isolated human monocytes with GLP-1 significantly decreased cell migration induced by both monocyte chemotactic protein-1 and by the protein known as regulated on activation, normal T cell expressed and secreted (RANTES) in a concentration-dependent manner. Furthermore, GLP-1 significantly decreased MMP-9 release from isolated human monocyte-derived macrophages.

Conclusions/interpretation Sitagliptin reduces plaque inflammation and increases plaque stability, potentially by GLP-1-mediated inhibition of chemokine-induced monocyte migration and macrophage MMP-9 release. The effects observed may provide potential mechanisms for how DPP-IV inhibitors could modulate vascular disease in high-risk patients with type 2 diabetes mellitus.

Keywords Atherosclerosis · Glucagon-like peptide-1 · Incretins · Plaque stability · Type 2 diabetes

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Abbreviations

DPP-IV	Dipeptidyl peptidase-IV
FAP	Fibroblast activation protein

GLP-1   Glucagon-like peptide-1
MAC-3   Macrophage-3 antigen
MCP-1   Monocyte chemotactic protein-1
MMP-9   Metalloproteinase-9
pNa   p-Nitroaniline
RANTES   Regulated on activation, normal T cell
          expressed and secreted
TIMP   Tissue inhibitor of metalloproteinases

Introduction

Dipeptidyl peptidase-IV (DPP-IV) inhibitors such as sita-gliptin, vildagliptin and saxagliptin are current treatment options for patients with type 2 diabetes mellitus [1]. These orally available drugs inhibit DPP-IV, thus increasing the half-life and plasma concentrations of the incretin hormone glucagon-like peptide-1 (GLP-1).

GLP-1 reduces blood glucose levels by enhancing insulin secretion from pancreatic beta cells on the one hand and by reducing glucagon release from alpha cells on the other. Besides these direct effects on the pancreas, GLP-1 reduces gastric emptying and appetite, improves peripheral glucose uptake, and limits hepatic glucose production, thus also indirectly contributing to glucose homeostasis. Moreover, recent data suggest that GLP-1 has direct effects on endothelial function [2, 3], inflammatory cell activation [4] and myocardial function [5]. Furthermore, the stable GLP-1 analogue exendin-4 stimulates the proliferation of human coronary artery endothelial cells in vitro through phosphoinositide 3-kinase/Akt- and endothelial nitric oxide synthase-dependent pathways, effects that are shared by both native GLP-1(7–36) and its major metabolite GLP-1(9–36) [6]. Given that patients with diabetes exhibit an increased propensity to developing vascular disease, with its sequelae of myocardial infarction and stroke, such beneficial effects of GLP-1 raise the question of to what extent increased GLP-1 concentrations might not only influence blood glucose levels, but also modulate vascular disease in this high-risk population.

DPP-IV degrades a large quantity of molecules, including multiple chemokines involved in the innate immune response, and DPP-IV inhibition has been shown to have direct inhibitory effects on CD4-positive T cell and monocyte migration, all of which are involved in atherogenesis [7–10]. In addition, clinical data suggest that DPP-IV inhibitors influence various cardiovascular risk factors associated with diabetes. For example, sitagliptin monotherapy significantly decreases triacylglycerol as well as NEFA levels and increases HDL-cholesterol in patients with type 2 diabetes [11]. Furthermore, a significant decrease in systolic blood pressure could be shown with sitagliptin in non-diabetic patients with mild to moderate hypertension [12].

Recently, the DPP-IV inhibitors des-fluoro-sitagliptin and alogliptin have been shown to decrease atherosclerosis quantitatively in mouse studies [9, 13]. However, little is known about the effects of DPP-IV inhibitors on plaque composition and on the stability of atherosclerotic plaques. The latter is in particular of importance in patients with type 2 diabetes, since lesions in these patients are usually more vulnerable, with a high burden of proinflammatory macrophages, a low collagen content and an increased susceptibility to rupture, which contributes to cardiovascular risk in these patients.

Therefore, the present study examined the effect of sitagliptin on lesion size, plaque inflammation and plaque collagen content in $Apoe^{-/-}$ mice. Furthermore, we investigated the action of GLP-1 on the chemokine-induced migration of human monocytes and human macrophage metalloproteinase-9 (MMP-9) levels, both crucial steps in atherogenesis and plaque destabilisation.

Methods

Animals C57BL/6 $Apoe^{-/-}$ mice were purchased from the Charles River Laboratories, Sulzfeld, Germany, at 7 weeks of age and were allowed to adjust to their new environment for 2 weeks. Mice were housed in the animal facility of the University of Ulm single-caged with free access to food and water and in a pathogen-free environment with a 12 h light/dark cycle. The protocol was approved by the Regierungspraesidium in Tuebingen, Germany, and all procedures were conducted in accordance with recommendations given by the animal care facility of the University of Ulm.

At 9 weeks of age, all animals were fed with a high-fat diet (Ssniff, Soest, Germany; based on research diets D12108) to accelerate the atherogenic process in this mouse model as previously reported [14, 15]. Animals were randomly assigned to two groups. One group received sitagliptin (kindly provided by MSD, Haar, Germany) for 12 weeks by premixing it with the high-fat diet at 1.1% (wt/wt approximately 576 mg sitagliptin/kg body weight; $n=10$). The other group served as controls and received only the high-fat diet ($n=10$). Sitagliptin at this dosage using the same route of administration has previously been used in mice to inhibit DPP-IV activity [16] and was chosen for this study in order to maximise the effects observed with DPP-IV inhibition, although the dosage is higher than in patients.

Body weight and individual food intake were monitored weekly. An OGTT was performed using the following protocol after 6 h of fasting the week before the mice were killed. A 20% (wt/vol) glucose solution (2 g glucose/kg body weight) was administered by oral gavage, and glucose levels were measured from tail vein blood with a glucometer (Precision Xceed; Abbott, Wiesbaden, Germany) at 0, 15, 30, 60, 90 and 120 min after the glucose challenge.

After 12 weeks on the high-fat diet, the mice were anaesthetised with an intraperitoneal injection of 2,2,2-tribromethanol (0.25 mg/g body weight) preceded by heparin injection, and were then killed by exsanguination from heart puncture.

DPP-IV activity measurement Aprotinin and EDTA (Sigma, Munich, Germany) were immediately added to the blood, which was processed later for DPP-IV activity measurement. DPP-IV activity was measured by modification of the enzymatic method established by Deacon et al as published previously [17–19]. Briefly, 5 μl of plasma was incubated in the presence of 3.3 mg H-Gly-Pro-p-nitroaniline (a DPP-IV substrate; Sigma, Munich, Germany), and the amount of p-nitroaniline (pNa) per minute generated by DPP-IV enzymatic activity was spectrophotometrically measured at 30, 60 and 120 min at a wavelength of 405 nm.

Lipid profile measurement Levels of total cholesterol, triacylglycerols, HDL-cholesterol and LDL-cholesterol were measured at the end of the feeding period in untreated plasma after 6 h of fasting in the central clinical laboratory of the University Hospital in Ulm.

Aorta collection and processing and lesion size evaluation The heart and the aorta from the aortic root to the iliac bifurcation were isolated from the mice after they had been killed. The base of the heart and the aortic arch until a point immediately after the left subclavian artery were cut and embedded in a tissue-freezing medium, frozen in liquid nitrogen and stored at −80°C until processing.

To evaluate plaque extension, the descending aortas, stored in formalin, were washed with PBS and with a 100% (vol./vol.) solution of propylene glycol, then stained with fresh filtered Oil Red O for 3 h and washed in 85% (vol./vol.) propylene glycol. The adventitia was stripped off, and the aortas were longitudinally cut and pinned on dishes filled with black silicon elastomers (the en face method). To further quantify the atherosclerotic lesions, formaldehyde-fixed sections of the aortic root and the aortic arch were rehydrated in PBS, washed in 100% (vol./vol.) propylene glycol, incubated for 20 min at 60°C with fresh filtered Oil Red O, and finally counterstained with haematoxylin.

The extent of the lesions was evaluated using image-processing software (Image Pro-plus; Media Cybernetics, Bethesda, MD, USA).

Immunohistochemistry and Picrosirius Red staining To quantify the macrophages inside the plaques, acetone-fixed sections of the aortic root and the aortic arch were stained with a goat anti-macrophage-3 antigen (MAC-3) mouse macrophage antibody (BD Biosciences, Heidelberg, Germany): sections were blocked with goat serum, incubated with the primary anti-MAC3 antibody, then incubated with a biotinylated secondary antibody, and finally counterstained with haematoxylin. To investigate plaque levels of MMP-9, further immunostainings were performed using a specific antibody against MMP-9 (Abcam, Cambridge, UK).

To assess the collagen fibre content of the lesions, formaldehyde-fixed sections of the aortic root and aortic arch were rehydrated and stained for 90 min in a 0.1% solution of Sirius Red in 1.2% picric acid solution. All sections were analysed under a light microscope (Nikon, Tokyo, Japan) with polarised films.

The amount of lipid and macrophage inclusion, as well as the total collagen fibre content and MMP-9 levels, was evaluated as the ratio of the positive area to the area of the lesion using image-processing software (Image Pro-plus).

Cell culture Human monocytes were isolated from the buffy coats of healthy volunteers by Ficoll Histopaque (Sigma) gradient centrifugation to obtain mononuclear cells, with subsequent negative selection of the monocytes by magnetic bead separation (Miltenyi Biotech, Teterow, Germany) according to the manufacturer's protocol. The purity of human monocytes was >95% as determined by flow cytometry.

In vitro cell migration assay The migration of isolated monocytes was determined as previously described [20]. Briefly, cells were cultured in serum-free media for 16 h. Monocytes (5×10^6 cells/ml) were pretreated for 30 min with GLP-1(7–36) (Sigma) at the concentrations indicated in serum-free medium and subsequently assayed for chemotaxis under serum-free conditions in a 48-well microchemotaxis chamber (Neuroprobe, Leamington Spa, Warwickshire, UK). Wells in the upper and lower chamber were separated by a polyvinylpyrrolidone-free polycarbonate membrane (pore size 5 μm; Whatman, Dassel, Germany) that had been preincubated with collagen. As a stimulus, 10 nmol/l monocyte chemotactic protein-1 (MCP-1; ReproTech, Rocky Hill, NJ, USA; or Sigma) or 100 ng/ml of the protein known as regulated on activation, normal T cell expressed and secreted (RANTES; ReproTech) was added to the lower wells of the chamber. After 3 h, migrated cells attached to the bottom face of the filter were stained. Cells were counted under the light microscope in five random high-power fields per well.

Zymographies Human monocytes were differentiated with RPMI + 5% (vol./vol.) human serum (PAA, Cölbe, Germany) and standard zymographies were performed as previously described [21].

Western blot analysis Standard western blot analysis was performed using antibodies against p-myosin light chain (p-MLC, Cell Signaling, Danvers, MA, USA).

Statistical analysis All data are presented as means±SD. Differences between the two groups were analysed by unpaired Student's *t* test or two-way ANOVA with Bonferroni's post hoc test where appropriate. Statistical significance was declared at $p<0.05$.

Results

Animal data and metabolic profile Nine-week-old male mice matched for baseline body weight were fed with a high-fat diet containing either sitagliptin or no further additives (control group). Weekly food intake and weight gain were similar in both groups (Table 1). To determine whether sitagliptin treatment effectively inhibited DPP-IV in our mouse model, the protease activity was measured in plasma samples on the day the mice were killed after 12 weeks of dietary treatment. Sitagliptin significantly reduced plasma DPP-IV activity by >85% (0.3 ± 0.6 vs 2.5 ± 1 nmol min^{-1} ml^{-1} pNa for sitagliptin vs controls; $p<0.05$) compared with controls. As expected, glucose tolerance was significantly ameliorated in mice treated with sitagliptin, with an AUC of 27.8 ± 3.5 mmol/l×h in sitagliptin and 31.7 ± 4.1 mmol/l×h in control mice ($p<0.01$; Table 1). Lipid levels were comparable in the two groups (Table 1).

Extent of atherosclerotic lesions To assess the effect of sitagliptin on overall extension of the lesions after 12 weeks on the high-fat diet, total plaque area in the descending aorta was quantified by the en face method. As shown in Fig. 1a, b, sitagliptin did not significantly influence lesion size compared with controls using the en face method ($8.1\pm3.5\%$ vs $5.1\pm2.5\%$ for sitagliptin vs controls; p=NS). This was confirmed by absolute values of plaque size in tissue sections of the aortic root (727 ± 139 μm^2 vs 716 ± 181 μm^2 for sitagliptin vs controls; p=NS; Fig. 1c, d) and the aortic arch (763±
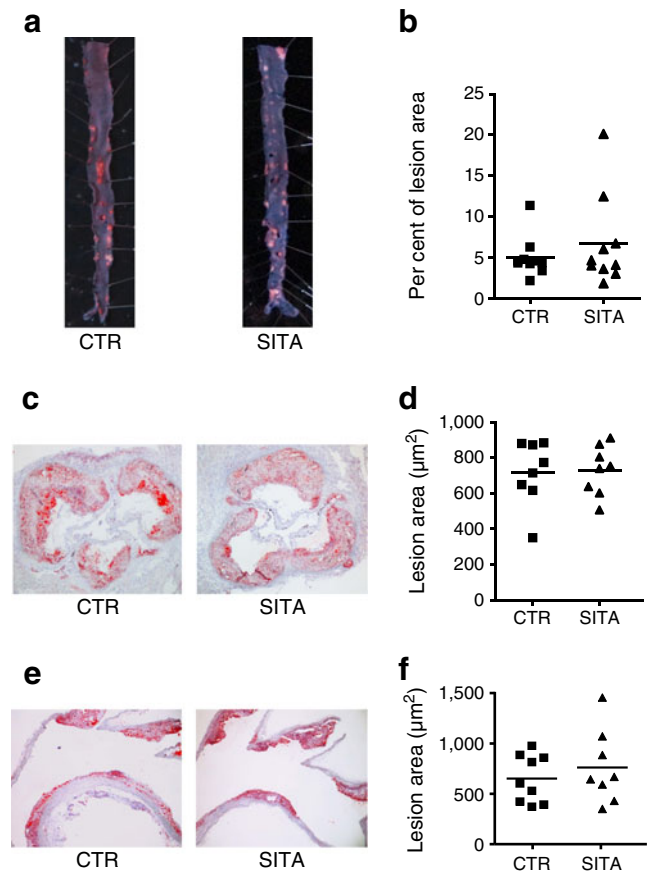
Fig. 1 Extension of atherosclerotic lesions in *Apoe*$^{-/-}$ mice on a high-fat diet with (SITA) or without (CTR) sitagliptin treatment. (**a,b**) Lesion extension in the descending aorta. (**c,e**) Oil Red O staining of aortic root (**c**) and aortic arch (**e**); the scatter plots (**d,f**) show quantification of the lesion area with means indicated by horizontal bars (p= NS between groups)

349 μm^2 vs 656 ± 211 μm^2 for sitagliptin vs controls; p=NS; Fig. 1e, f), where no differences in lesion extension could be found between the two groups.

Macrophage content in arteriosclerotic lesions To assess whether sitagliptin influences lesion composition, we next evaluated plaque macrophage content using MAC-3 as a cell type-specific marker. Compared with controls, sitagliptin treatment significantly reduced macrophage content in the atherosclerotic lesions of both the aortic root and the aortic arch (0.3 ± 0.2 vs $0.9\pm0.5\%$ and 0.4 ± 0.3 vs $1.3\pm1.0\%$ for sitagliptin vs controls in the aortic root and aortic arch, respectively; $p<0.05$; Fig. 2), suggesting that DPP-IV inhibitor treatment modulated the inflammatory cell composition of the plaques.

MMP-9 content in arteriosclerotic lesions After observing less macrophage infiltration in the atherosclerotic lesions of sitagliptin-treated mice, and since plaque macrophages release matrix-degrading enzymes such as MMP-9, we next investigated plaque MMP-9 content. As depicted in Fig. 3,
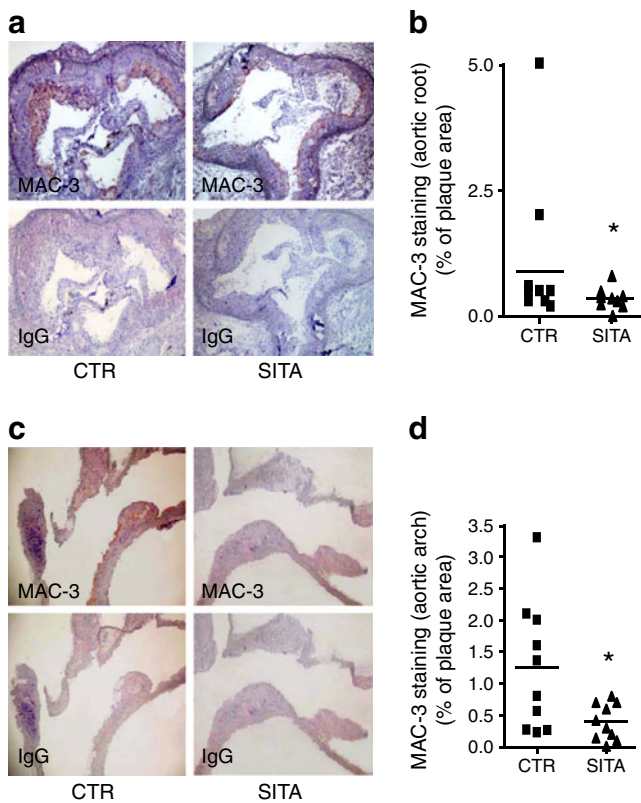
Table 1 Characteristics of *Apoe*$^{-/-}$ mice treated for 12 weeks with a high-fat diet and sitagliptin (SITA) or a high-fat diet alone (CTR)

Variable	CTR	SITA	*p* value
Weight (g)	31.2 ± 2.9	29.4 ± 1.2	NS
Food intake (g/week)	23.6 ± 3.1	22.4 ± 0.8	NS
Total cholesterol (mmol/l)	14.3 ± 2.2	13.0 ± 1.2	NS
Triacylglycerols (mmol/l)	0.89 ± 0.31	1.36 ± 0.29	NS
LDL-cholesterol (mmol/l)	9.4 ± 1.8	10.7 ± 0.5	NS
Baseline glucose (mmol/l)	11.3 ± 2.8	9.8 ± 1.5	NS
OGTT$_{AUC}$ (mmol/l × h)	31.7 ± 4.1	27.8 ± 3.5	<0.01
DPP-IV activity (nmol min^{-1} ml^{-1} pNa)	2.5 ± 1.0	0.3 ± 0.6	<0.05

Data are displayed as means±SD, n=10

OGTT$_{AUC}$, area under the curve of the OGTT

Fig. 2 Plaque macrophage content in $Apoe^{-/-}$ mice on a high-fat diet with (SITA) or without (CTR) sitagliptin treatment. Representative tissue sections from the aortic root (**a**) or aortic arch (**c**) stained with anti-MAC3 antibodies are displayed. Adjacent sections stained with similar concentrations of type- and class-matched IgG served as controls. (**b**,**d**) Quantification of plaque macrophage content is displayed as scatter plots with means indicated by horizontal bars ($n=9$ or 10, $*p<0.05$ for SITA vs CTR)

Fig. 3 Plaque MMP-9 levels in $Apoe^{-/-}$ mice on a high-fat diet with (SITA) or without (CTR) sitagliptin treatment. Representative tissue sections from the aortic root (**a**) or aortic arch (**c**) stained with anti-MMP-9 antibodies are displayed. Adjacent sections stained with similar concentrations of type- and class-matched IgG served as controls. (**b**,**d**) Quantification of plaque MMP-9 levels is displayed as scatter plots with means indicated by horizontal bars ($n=8$ or 9, $**p<0.01$ for SITA vs CTR). High-power views of representative pictures are included as insets

treatment with sitagliptin significantly reduced plaque MMP-9 content compared with controls in lesions located in both the aortic root and the aortic arch (9 ± 7 vs $28\pm15\%$ and 6 ± 3 vs $15\pm7\%$ for sitagliptin vs controls in the aortic root and aortic arch, respectively; $p<0.01$).

Collagen content in arteriosclerotic lesions Since MMP-9 degrades extracellular matrix, thus directly influencing plaque collagen content and plaque stability, we next assessed the effect of sitagliptin on types I and III collagen in lesions from these mice. Picrosirius Red staining revealed that sitagliptin significantly increased type I and III collagen content in the aortic root and aortic arch compared with controls (1.2 ± 0.6 vs $0.5\pm0.3\%$ in the aortic root [$p<0.01$] and 2.6 ± 0.7 vs $1.1\pm0.9\%$ in the aortic arch [$p<0.05$], respectively for sitagliptin vs controls; Fig. 4).

GLP-1 inhibits chemokine-induced migration of human monocytes and macrophage MMP-9 levels To investigate whether the observed decrease in plaque macrophage
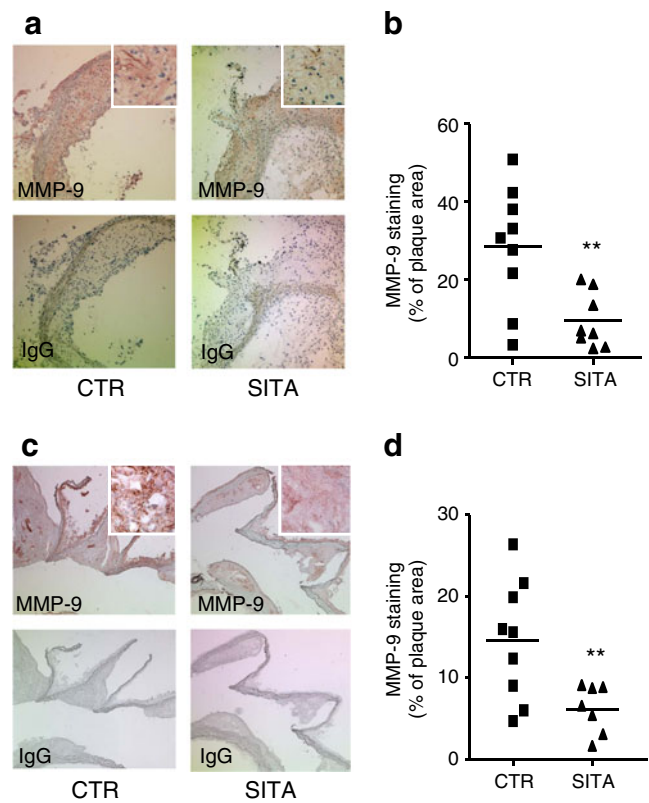
content in sitagliptin-treated mice might be caused by direct effects of GLP-1 on monocyte migration, we performed in vitro migration experiments using a modified Boyden chamber. As shown in Fig. 5a, stimulation of isolated human monocytes with MCP-1 significantly increased cell migration (2.3 fold; $p<0.01$; $n=8$); pretreatment of cells with GLP-1(7–36) for 30 min reduced this effect in a concentration-dependent manner by a maximal 69% to a 1.4 ± 0.5 fold induction at 10 nmol/l GLP-1 ($p<0.05$ compared with MCP-1 treated cells; $n=8$). Similar effects were seen when RANTES was used as an inducer of monocyte migration (Fig. 5b) suggesting that the action of GLP-1 on monocyte migration is independent of the chemokine used. Furthermore, heat-inactivated GLP-1 did not inhibit chemokine-induced monocyte migration (Fig. 5b).

To further elucidate the cellular mechanisms involved in the inhibition of cell migration, we examined the effect of GLP-1 on MLC phosphorylation in human monocytes. As shown in Fig. 5c, MCP-1 induces phosphorylation of MLC, and pretreatment with GLP-1 inhibited this MCP-1-induced phosphorylation of MLC in a concentration-dependent manner.
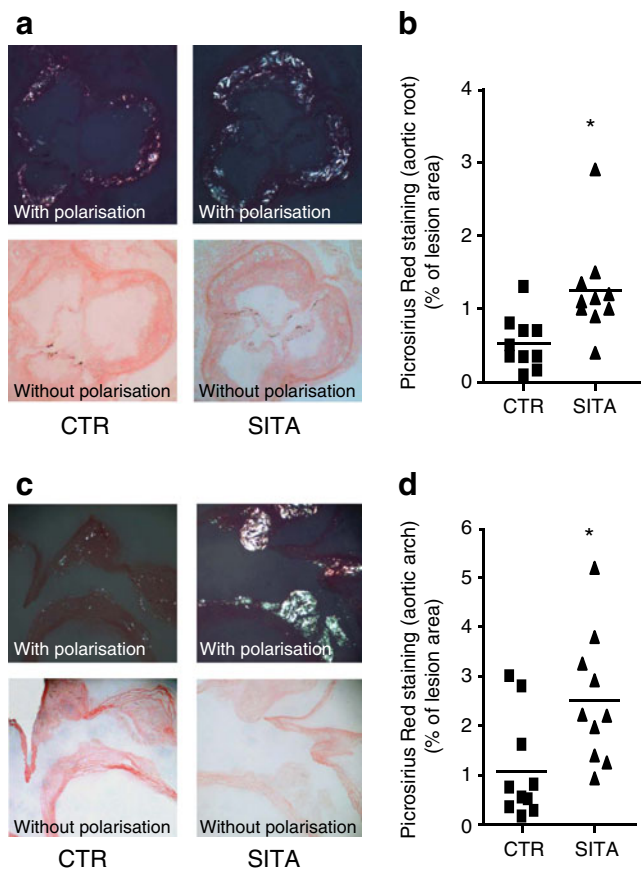
Fig. 4 Plaque collagen content in *Apoe*^{−/−} mice on a high-fat diet with (SITA) or without (CTR) sitagliptin treatment. Representative tissue sections from the aortic root (**a**) and aortic arch (**c**) show Sirius Red staining without or with polarisation to demonstrate the collagen content. (**b**,**d**) Quantification of plaque collagen content is displayed as scatter plots with means indicated by horizontal bars ($n=10$ per group, $*p<0.05$ for SITA vs CTR)

In addition, we investigated the effects of GLP-1 on the MMP-9 content of human monocyte-derived macrophages using gelatine zymography to determine whether the decrease in plaque MMP-9 content observed in sitagliptin-treated mice might be caused by direct effects of GLP-1 on macrophage MMP-9 levels. As shown in Fig. 5d, GLP-1 inhibited MMP-9 production of monocyte-derived macrophages in a concentration-dependent manner.

Discussion

The present study demonstrates that sitagliptin reduces plaque macrophage infiltration and MMP-9 levels, thus leading to an increase in plaque collagen content. The data suggest that DPP-IV inhibitor treatment may stabilise arteriosclerotic lesions in this mouse model of vascular disease despite not having an effect on lesion size.
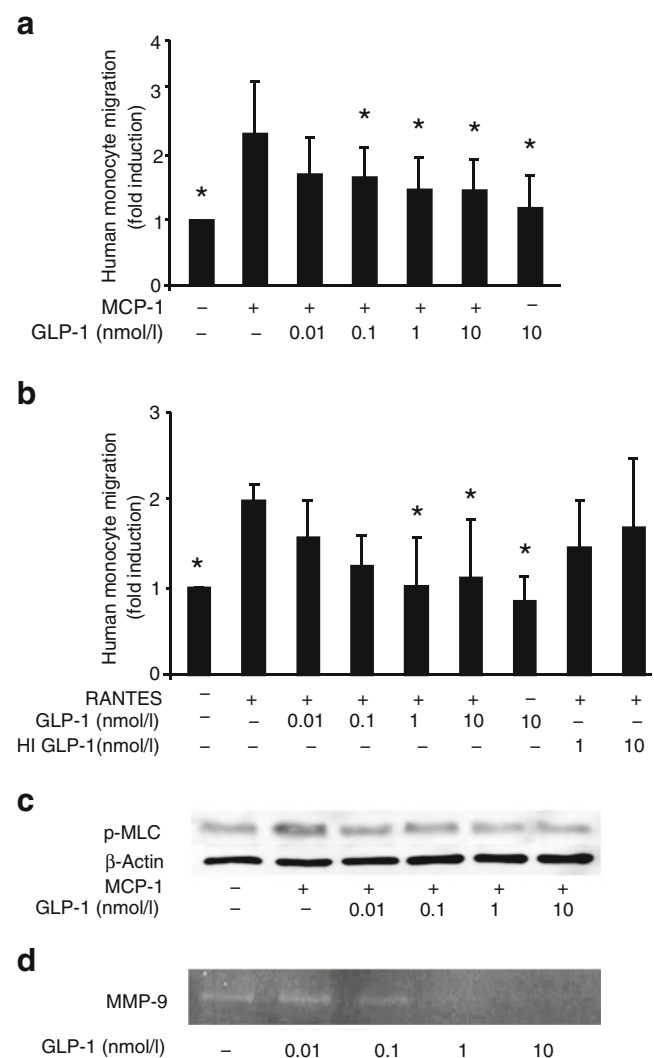

Fig. 5 (**a**,**b**) GLP-1 reduces chemokine-induced migration of human monocytes. Isolated human monocytes were pretreated with GLP-1 for 30 min at the concentrations indicated before migration experiments using MCP-1 (10 nmol/l) (**a**) or RANTES (100 ng/ml) (**b**) were performed in a modified Boyden chamber. Data are expressed as fold induction of unstimulated cells. Bars represent means ± SD ($n=8$ for MCP-1, $n=5$ for RANTES). HI GLP-1, heat-inactivated GLP-1. (**c**) GLP-1 reduces MCP-1-induced phosphorylation of MLC in isolated human monocytes. Cells were pretreated with GLP-1 for 30 min at the concentrations indicated before stimulation with MCP-1 (10 nmol/l). Western blot analysis for p-MLC was performed after 5 min. ß-Actin protein level served as a loading control. Representative results from three independent experiments are shown. (**d**) GLP-1 reduces MMP-9 content of human monocyte-derived macrophages. Cells were treated with GLP-1 overnight at the concentrations indicated and standard zymography was performed on the supernatant fractions. Representative results from three independent experiments are shown

GLP-1 is a peptide hormone that is produced in the distal gastrointestinal tract. It is rapidly inactivated by DPP-IV and has been shown to enhance glucose-dependent insulin secretion and production, as well as to increase insulin sensitivity in diabetic patients [22]. GLP-1 analogues and DPP-

IV inhibitors, which increase GLP-1 plasma concentrations, are current treatment options for patients with diabetes mellitus [23]. In addition to its well-known effects on glucose metabolism, GLP-1 has recently been shown to exhibit beneficial effects on the cardiovascular system [24]. Moreover, there is evidence of anti-inflammatory effects of GLP-1 in different tissues [25], as well as a role for DPP-IV in the modulation of the inflammatory processes, including the degradation of chemokines and direct effects of leucocyte chemotaxis and activation [8–10, 26]. More recently, the DPP-IV inhibitors des-fluoro-sitagliptin and alogliptin have been shown to reduce atherosclerosis in mouse studies [9, 13]. Our study now extends the knowledge on the role of GLP-1 and DPP-IV inhibitors in the cardiovascular system by demonstrating that sitagliptin treatment decreases plaque macrophage content and stabilises arteriosclerotic lesions in $Apoe^{-/-}$ mice, while GLP-1 directly inhibits monocyte migration and macrophage MMP-9 production in vitro. However, clinical studies are needed to show whether this increase in collagen content in mice translates into a plaque-stabilising effect in humans.

In our mouse model, the dose of sitagliptin employed was effective in inhibiting DPP-IV activity, leading to an improvement in the metabolic profile, comparable to what has previously been reported in patients [11]. Despite the improvement in glucose tolerance in sitagliptin-treated mice, the extent of atherosclerotic lesions did not differ compared with controls. However, we observed a significant reduction in plaque macrophage infiltration, suggesting that sitagliptin affects cellular plaque composition in these mice. Plaque macrophages locally release matrix-degrading enzymes such as MMP-9, thus influencing plaque collagen content and plaque stability. Therefore, the decrease in plaque MMP-9 content and the increase in collagen type I and III observed here may be the consequence of a reduction in macrophage infiltration in the plaque. Lesion size, plaque macrophage infiltration, plaque MMP-9 staining and plaque collagen content were consistent in both the aortic root and aortic arch.

However, our mouse model is limited by the fact that it does not allow quantification of the necrotic core of the plaques. Our in vitro data suggest that this decrease in plaque monocyte/macrophage content upon sitagliptin treatment may be due to a direct inhibition of chemokine-induced monocyte migration by GLP-1. Even though we could demonstrate reduced DPP-IV activity and an improved metabolic profile in mice treated with sitagliptin as indirect evidence of elevated GLP-1 levels, a limitation of this study is that GLP-1 levels have not been measured in our mice and can therefore not be directly associated to the vascular effects observed in this study. Furthermore, we cannot exclude the involvement of other metalloproteinases and their antagonists, the tissue inhibitors of metalloproteinases (TIMPs) in plaque stabilisation in sitagliptin-treated mice, in particular given that TIMP3

has recently been shown to be involved in plaque stabilisation and monocyte migration [27, 28]. In addition, we cannot exclude the fact that improved glucose tolerance in sitagliptin-treated mice may influence vascular chemokine or adhesion molecule levels or secondary effects on cell migration. Another limitation of this study is that the recently described beneficial cardiovascular effects of both GLP-1-mediated and direct effects of DPP-IV inhibition on endothelial nitric oxide synthase have not been investigated in our model [6, 29].

Recently, Brokopp et al demonstrated that fibroblast activation protein (FAP), a member of the prolyl peptidase family, is induced by inflammation in thin-cap fibroatheromata and degrades type I collagen [30]. Given that FAP activity can be inhibited by high concentrations of sitagliptin [31], we cannot exclude FAP inhibition as an additional cause for the decreases in plaque collagen content observed in our study.

Another study showed that treatment with the GLP-1 receptor agonist exendin-4 was associated with a reduction in monocyte adhesion on endothelial cells and a decrease in lesion size in $Apoe^{-/-}$ mice [32]. More recently, Matsubara et al and Shah et al have suggested a decrease in atherosclerosis with the DPP-IV inhibitors des-fluoro-sitagliptin and alogliptin [9, 13]. Although the decreases in monocyte adhesion and plaque inflammation mentioned are in line with our finding of reduced monocyte/macrophage infiltration in plaques, we did not find a reduction in lesion size after sitagliptin treatment. This discrepancy may have occurred for several reasons.

First, although GLP-1 and glucose-dependent insulinotropic polypeptide are endogenous substrates of DPP-IV and are mostly responsible for the effects on glucose metabolism, other DPP-IV substrates include various chemokines and peptides [10, 33], some of which play a critical role in atherogenesis. Thus, the difference between the studies may stem from different effects of GLP-1 analogues and DPP-IV inhibitors on these mediators or may be dependent on the choice of the DPP-IV inhibitor and the doses employed. The dose used in our study was approximately three times as high as that used by Matsubara et al, and the different results in terms of plaque size may originate from the differential effects on the degradation of proatherogenic mediators by DPP-IV [13].

Second, GLP-1 analogues are specific stimulators of the GLP-1 receptor, and GLP-1, which is increased upon DDP-IV inhibitor treatment, has been shown to exhibit various effects independent of the established GLP-1 receptor [34]. Further studies are warranted to examine the extent to which the effects seen in our study are independent of the known GLP-1 receptor.

In conclusion, our data demonstrate that 12 weeks of treatment with sitagliptin reduces plaque macrophage infiltration as

well as plaque MMP-9 levels, thus leading to an increase in the collagen fibre content of atherosclerotic lesions in *Apoe*$^{-/-}$ mice on a high-fat diet. Such findings are of potential clinical importance: lesions from patients with diabetes mellitus exhibit a high burden of inflammatory cells and are more vulnerable than lesions in individuals without diabetes [35–37]. Therefore, increasing plaque stability by influencing vascular inflammation with DPP-IV inhibitors could be an interesting concept to modulate vascular disease in patients with type 2 diabetes. However, whether the positive effects of sitagliptin on plaque inflammation and stability observed in this murine study translate into a reduction in cardiovascular morbidity and mortality in patients with type 2 diabetes is still unclear. Retrospective analyses of phase II and III studies in saxagliptin-treated patients with diabetes suggest a beneficial effect of DPP-IV-inhibitor treatment on cardiovascular events [38], but only the ongoing prospective controlled outcome trials such as Trial Evaluating Cardiovascular Outcomes with Sitagliptin (TECOS), Saxagliptin Assessment of Vascular Outcomes Recorded in Patients with Diabetes Mellitus (SAVOR-TIMI 53) or Cardiovascular Outcome Study of Linagliptin Versus Glimepiride in Patients with Type 2 Diabetes (CAROLINA) will finally answer this important question.

Duality of interest N. Marx served as a speaker for Berlin Chemie, Novo Nordisk, Merck Sharp and Dohme, Boehringer Ingelheim and Glaxo Smith Kline and an advisor to Glaxo Smith Kline, Merck Sharp and Dohme, Boehringer Ingelheim, Bristol-Myers Squibb and Astra Zeneca. The other authors declare that there is no duality of interest associated with this manuscript. Specifically, the authors declare that there was no involvement of MSD in the experiments, data analysis or manuscript preparation.

Contribution statement All authors have taken part in the conception and design of this work or the interpretation of the data as well as in the preparation of the manuscript. All authors have given final approval of the version to be published.

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