Cell. Mol. Life Sci. (2010) 67:3549–3555
DOI 10.1007/s00018-010-0396-5

Cellular and Molecular Life Sciences

RESEARCH ARTICLE

Glucagon-like peptide-1(1-37) inhibits chemokine-induced migration of human CD4-positive lymphocytes

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Received: 31 October 2009 / Revised: 12 April 2010 / Accepted: 29 April 2010 / Published online: 21 May 2010

Abstract The present study examined the effect of GLP-1(1-37) on chemokine-induced CD4-positive lymphocyte migration as an early and critical step in atherogenesis. Pretreatment with GLP-1(1-37) reduced the SDF-induced migration of isolated human CD4-positive lymphocytes in a concentration-dependent manner. Similar effects were seen when RANTES was used as a chemokine. GLP-1(1-37)'s effect on CD4-positive lymphocyte migration was mediated through an early inhibition of chemokine-induced PI-3 kinase activity. Downstream, GLP-1(1-37) inhibited SDF-induced phosphorylation of MLC and cofilin and limited f-actin formation as well as ICAM3 translocation. Furthermore, exendin-4 inhibited SDF-induced migration of CD4-positive lymphocytes similarly to GLP-1(1-37), and transfection of these cells with GLP-1 receptor siRNA abolished GLP-1(1-37)'s action on chemokine-induced ICAM3 translocation, suggesting an effect mediated via the GLP-1 receptor. Thus, GLP-1(1-37) inhibits chemokine-induced CD4-positive lymphocyte migration by inhibition of the PI3-kinase pathway and via the GLP-1 receptor. This effect provides a potential novel mechanism for how GLP-1(1-37) may modulate vascular disease.

Keywords GLP-1 · CD4-positive lymphocytes · Migration · Chemokines · Atherosclerosis

Introduction

According to our current understanding, atherogenesis is an inflammatory process in the vessel wall, involving inflammatory cells such as monocytes and CD4-positive lymphocytes. The latter are considered to be of critical importance for local vascular inflammation. CD4-positive lymphocytes are attracted by chemotactic proteins such as RANTES and SDF-1 and enter the vessel wall as naïve TH0 cells. In the subendothelium, these cells then encounter antigens such as oxidized LDL and differentiate into TH1 cells, subsequently releasing pro-inflammatory mediators such as TNF-α and interferon-γ (IFNγ). These cytokines then activate other cells such as endothelial cells, macrophages, and vascular smooth muscle cells, thus promoting the inflammatory process of atherogenesis in the vessel wall [1, 2]. Lesions from patients with diabetes mellitus, a high-risk population for vascular disease, exhibit a high burden of inflammatory cells [3]. Moreover, experimental studies have shown that a reduction in CD4-positive lymphocyte recruitment hampers lesion development and plaque formation [4, 5]. Still, most of these studies targeted the effect of T-cell-specific chemokines, but hitherto little is known about modulatory effects on CD4-positive lymphocyte migration.

The untruncated GLP-1(1-37) is a 37 amino acid hormone that is secreted from the human pancreas and to a lesser extent from intestinal cells [6]. Whereas multiple metabolic and nonmetabolic effects of the truncated GLP-1 have been described [7–11], the physiological role of untruncated GLP-1(1-37) remains incompletely understood.

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GLP-1(1-37) is a less potent GLP-1 receptor agonist [12, 13] than truncated GLP-1 and has been shown to modulate rat parietal cell function [12]. Recently, GLP-1(1-37) has been shown to convert intestinal epithelial cells into insulin-producing cells [14], thus suggesting a new therapeutic tool for the treatment of diabetes mellitus. We hypothesized that GLP-1(1-37) could also play a role in modulating vascular inflammation in patients with diabetes mellitus who have an increased propensity to develop a diffuse and severe atherosclerosis. However, to date nothing is known about the role of GLP-1(1-37) on vascular cells such as CD4-positive lymphocytes and potential effects on T-cell migration as a critical step in lesion development. Therefore, the present study examined the effect of GLP-1(1-37) on chemokine-induced CD4-positive lymphocyte migration as one of the early and critical steps in atherogenesis.

Materials and methods

Cell culture

Human CD4-positive lymphocytes were isolated from freshly drawn blood of healthy volunteers by Ficoll-Histopaque (Sigma) gradient centrifugation to obtain mononuclear cells (PBMCs) and subsequent negative selection of CD4-positive T-cells by magnetic bead separation (Miltenyi Biotech) as described by the manufacturer's protocol. The purity of CD4-positive T cells was >97% as determined by flow cytometry.

In vitro cell migration assay

After isolation, CD4-positive lymphocytes were cultured in serum-free media for 16 h. T-cell chemotaxis was assayed under serum-free conditions in a 48-well microchemotaxis chamber (Neuroprobe). Wells in the upper and lower chamber were separated by a polyvinylpyrrolidone-free polycarbonate membrane (pore size 5 μm; Costar). CD4-positive cells at a density of 5×10^6/ml were pretreated for 30 min with GLP-1(1-37) (Sigma and Bachem) or exendin-4 (GenScript) at the concentrations indicated before 3 h of incubation with SDF-1 or RANTES (Sigma). Migrated cells attached to the bottom face of the filter, which had been preincubated with collagen, and were stained and counted under the light microscope. Cells were counted in five random high-power fields per well. Migration experiments were performed with GLP-1(1-37) from two different suppliers to exclude the possibility that the effects seen might be due to contamination by a biologically active substance. Furthermore, migration experiments were performed with a scrambled GLP-1(1-37)mod, which consists of the same amino acids as GLP-1(1-37) but in a random order (Phe-Ala-Gly-Ile-Arg-Trp-Gly-His-Asp-Arg-Val-Ala-Ala-Gln-Lys-Thr-Thr-Leu-Asp-His-Phe-Ser-Ser-Ser-Tyr-Phe-Gly-Val-Glu-Ala-Lys-Glu-Glu-Gly-Leu-Glu-Glu, Thermo Fisher Scientific).

Western blot analysis

Standard Western blot analysis was performed using anti-human GLP-1 receptor antibodies (Upstate), antibodies against *p*-cofilin and *p*-MLC (Cell Signaling).

Phosphatidylinositol kinase assay

After isolation, human T-cells were incubated for 16 h in RPMI medium without serum. Cells pretreated for 30 min with and without GLP-1(1-37) were stimulated with 100 ng/mL SDF-1. Standard PI 3-kinase activity assays were performed [15] using goat anti-human p85 (Santa Cruz) antibodies.

F-actin staining

After 30 min pretreatment with GLP-1(1-37), CD4-positive lymphocytes (2×10^6 cells/mL) were treated with SDF-1 at 100 ng/mL for times indicated. After stimulation, cells were fixed in 3.7% (wt/vol) paraformaldehyde in PBS, pH 7.4, and then washed at room temperature in TBS (50 mmol/L Tris-HCl, pH 7.6, 150 mmol/L NaCl, 0.1% NaN_3). For intracellular staining of F-actin, cells were permeabilized with 0.1% Triton X-100 for 10 min before application of the first antibody. Cell suspensions were incubated for 30 min in PBS with FITC-conjugated phalloidin (Sigma). Flow cytometry analysis was performed in a FACScan cytofluorometer (Becton-Dickinson) and induction of F-actin was measured.

ICAM3 staining

Immunofluorescence staining was performed as described before [15]. Briefly, $1–2 \times 10^6$ CD4-positive lymphocytes were incubated in special four-well plates (Costar, Cambridge, MA, USA) in a final volume of 500 μl complete medium on coverslips coated with collagen. Before treatment with SDF-1 (100 ng/mL, 30 min), cells were incubated with GLP-1(1-37) for 30 min. Cells were then fixed with 3.7% (wt/vol) paraformaldehyde in PBS, pH 7.4, at room temperature and rinsed in TBS. Cells were incubated with a specific antibody against ICAM3 (mouse anti-human ICAM3, Caltag), and after washing with PBS, carboxymethylindocyanine 3-Cy3-coupled (Dianova) goat anti-mouse IgGs were added as secondary antibodies (dilution 1:1,000) for 45 min. After washing again slides were stained with DAPI (nuclei staining). Images were

recorded with a fluorescence microscope (Leica) and cells in five randomly selected fields (~100 cells) were analyzed. ICAM3 translocation was considered to be present when a clear clustering of ICAM3 at the uropod was visible.

Transfection of siRNA

Specific and negative siRNAs were purchased from Invitrogen. Negative siRNA refers to negative control Validated Stealth (Invitrogen) and has no homology to any known human gene sequence. siRNA transfection was performed employing human CD4-positive lymphocytes. Cells were isolated from blood and incubated for 1 h in RPMI 1640 medium with 5% human serum. T-cells were transfected with GLP-1 receptor siRNA (200 nM) using the human T cell-nucleofector kit (Amaxa Biosystems) according to the manufacturer's protocol. After transfection, cells were plated in 12-well plates with RPMI 1640 medium containing 5% human serum and incubated for 48 h. Untreated cells, amaxa nucleofector solution, and negative siRNA controls were established in parallel.

Statistical analysis

Results of the experimental studies are reported as mean ± standard deviation (SD). Differences were analyzed by one-way ANOVA followed by the appropriate post-hoc test. A p value <0.05 was regarded as significant.

Results

GLP-1(1-37) reduces chemokine-induced CD4-positive lymphocyte migration

To examine the effect of GLP-1(1-37) on CD4-positive lymphocyte migration, cells were stimulated with SDF-1 in the absence or presence of GLP-1(1-37), and lymphocyte migration was assessed in a modified Boyden chamber. SDF-1 treatment significantly induced cell migration by 3.4 ± 1.7-fold ($p < 0.01$; $n = 9$), and pretreatment of cells with GLP-1(1-37) for 30 min reduced this effect in a concentration-dependent manner to a maximum 1.1 ± 0.7-fold induction at 1 nmol/L GLP-1(1-37) ($p < 0.01$ compared with SDF-1-treated cells; $n = 9$) (Fig. 1a). Similar effects were obtained when RANTES was used as a stimulus to induce CD4-positive lymphocyte migration (Fig. 1b), suggesting that the action of GLP-1(1-37) on lymphocyte migration is independent of the stimulus employed. To exclude that the phenomenon observed is caused by unspecific effects of a 37 amino acid peptide,

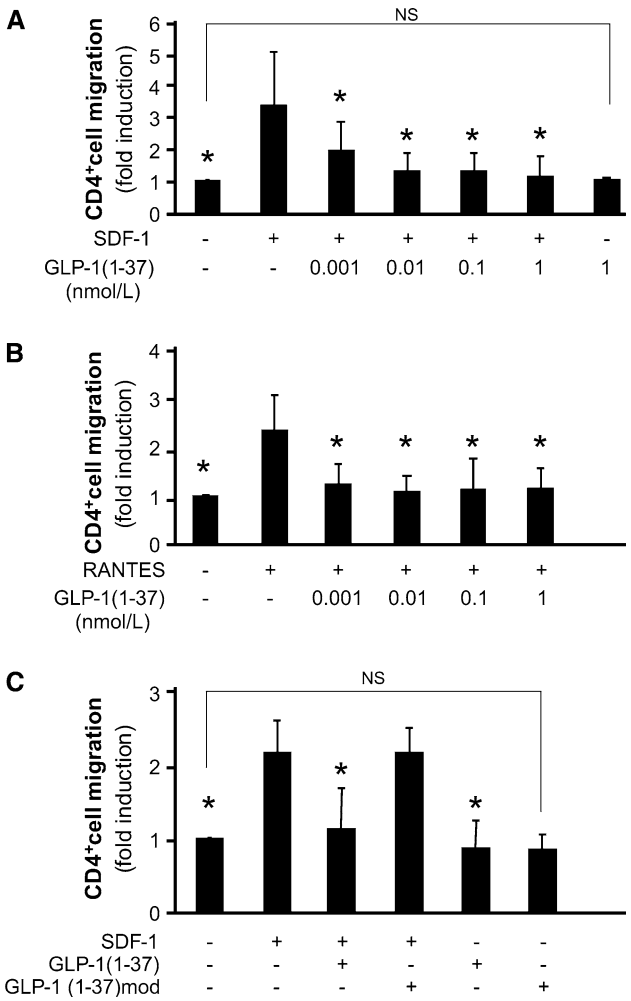

Fig. 1a–c GLP-1(1-37) reduces chemokine-induced migration of CD4-positive lymphocytes. Human CD4-positive lymphocytes were pretreated with GLP-1(1-37) for 30 min at concentrations indicated before migration experiments using **a** SDF-1 (100 ng/mL) or **b** RANTES (100 ng/mL) were performed in a modified Boyden chamber. Data are expressed as fold induction of unstimulated cells. *Bars* represent mean ± SD ($n = 9$ for SDF-1; $n = 11$ for RANTES); *$p < 0.01$ compared with chemokine-stimulated cells. GLP-1(1-37) alone did not induce CD4-positive lymphocyte migration compared to unstimulated cells. **c** Scrambled GLP-1(1-37) [GLP-1(1-37)mod] does not inhibit SDF-1-induced CD4-positive lymphocyte migration. Human CD4-positive lymphocytes were pretreated with 1 nM GLP-1(1-37) and 1 nM GLP-1(1-37)mod for 30 min before migration experiments using SDF-1 (100 ng/mL) were performed in a modified Boyden chamber. Data are expressed as fold induction of unstimulated cells. *Bars* represent mean ± SD ($n = 5$); *$p < 0.01$ compared with chemokine-stimulated cells. GLP-1(1-37)mod alone did not induce CD4-positive lymphocyte migration compared to unstimulated cells

migration experiments were performed using scrambled GLP-1(1-37) [GLP-1(1-37)mod], which consists of the same amino acids as GLP-1(1-37) but in a random order. In these experiments, GLP-1(1-37)mod did not inhibit SDF-1-induced CD4-positive lymphocyte migration (Fig. 1c).

Neither GLP-1(1-37) nor GLP-1(1-37)mod induced CD4-positive lymphocyte migration by themselves.

Moreover, GLP-1(1-37) did not affect cell viability and had no effect on the expression of the chemokine receptor CXCR4 as assessed by flow cytometry (data not shown).

GLP-1(1-37) reduces SDF-1-induced activation of PI-3 kinase in CD4-positive lymphocytes

Activation of PI-3 kinase is a critical step in chemokine-induced T-cell migration downstream of the respective chemokine receptor [16]. Therefore, we examined the effect of GLP-1(1-37) on PI-3 kinase activity. As demonstrated in Fig. 2, GLP-1(1-37) significantly limited SDF-1-induced PI-3 kinase activity in a concentration-dependent manner, suggesting that GLP-1(1-37) modulates a very upstream step in the chemokine-activated signaling cascade.

GLP-1(1-37) inhibits SDF-1-induced phosphorylation of cofilin and MLC

Downstream of PI3-kinase, stimulation of CD4-positive lymphocytes with chemokines leads to serine phosphorylation of cofilin thus rendering this mediator of actin filament depolymerization inactive during migration. In addition, MLC is phosphorylated upon chemokine stimulation to promote cell contraction. Using Western blot analysis, we demonstrate that GLP-1(1-37) inhibits both SDF-1-induced phosphorylation of cofilin and MLC (Fig. 3a) in a concentration-dependent manner, thus interfering with additional signaling processes during cell migration.

GLP-1(1-37) reduces f-actin formation in CD4-positive lymphocytes

Next we investigated the effect of GLP-1(1-37) on F-actin formation in CD4-positive lymphocytes, a crucial mechanism in cell polarization during migration [17, 18]. As shown in Fig. 3b, GLP-1(1-37) significantly reduced SDF-1-induced F-actin formation as assessed by flow cytometry analysis.

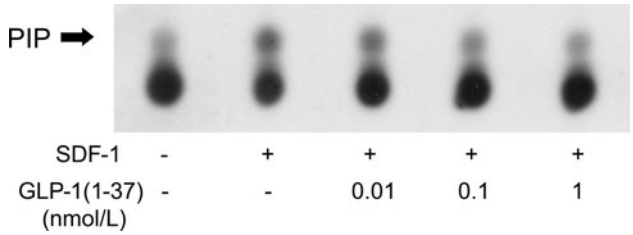

Fig. 2 GLP-1(1-37) inhibits SDF-1-induced PI 3-kinase activity. Human CD4-positive lymphocytes were pretreated with GLP-1(1-37) for 30 min at concentrations indicated before stimulation with SDF-1 (100 ng/mL). After 5 min PI 3-kinase activity assays were performed. *Specific dots* are labelled with an *arrow* (PIP). Four independent experiments showed similar results

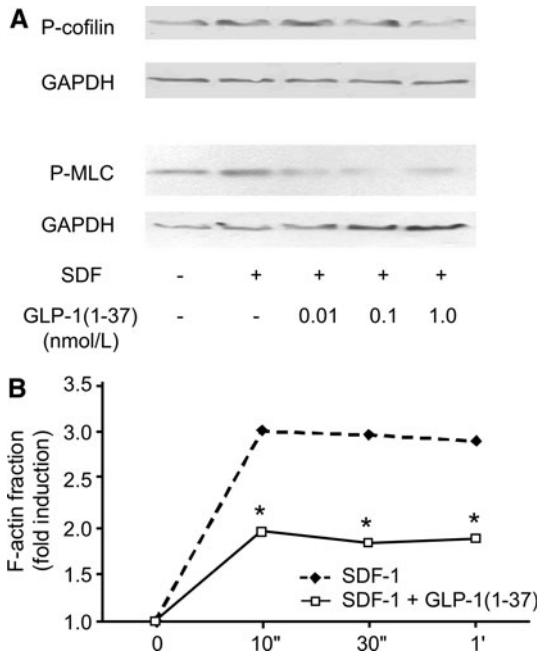

Fig. 3 **a** GLP-1(1-37) reduces SDF-1-induced phosphorylation of cofilin and MLC in CD4-positive lymphocytes. Isolated lymphocytes were pretreated with GLP-1(1-37) for 30 min at concentrations indicated before stimulation with SDF-1. Western blot analysis for *p*-cofilin and *p*-MLC was performed after 5 min. GAPDH protein expression served as a loading control. Representative results of four independent experiments are shown. **b** GLP-1 reduces SDF-1-induced F-actin formation. CD4-positive lymphocytes were pretreated with GLP-1 for 30 min (1 nmol/L) before stimulation with SDF-1. Actin polymerization was determined by flow cytometry at times indicated. *Dots* represent means. $*p < 0.05$ for comparison between groups at times indicated; $n = 3$

GLP-1(1-37) inhibits ICAM3 translocation in CD4-positive lymphocytes

Since ICAM3 translocation to the uropod of migrating lymphocytes is a critical step in cell movement [19], we next examined the effect of GLP-1(1-37) on ICAM3 translocation by means of immunofluorescence staining. As shown in Fig. 4, GLP-1(1-37) significantly reduced SDF-1-induced ICAM3 translocation to the uropod.

The effect of GLP-1(1-37) on chemokine-induced migration of human CD4-positive lymphocytes is mediated by the GLP-1 receptor

Next we examined whether the effect of GLP-1(1-37) on cell migration is mediated via the known GLP-1

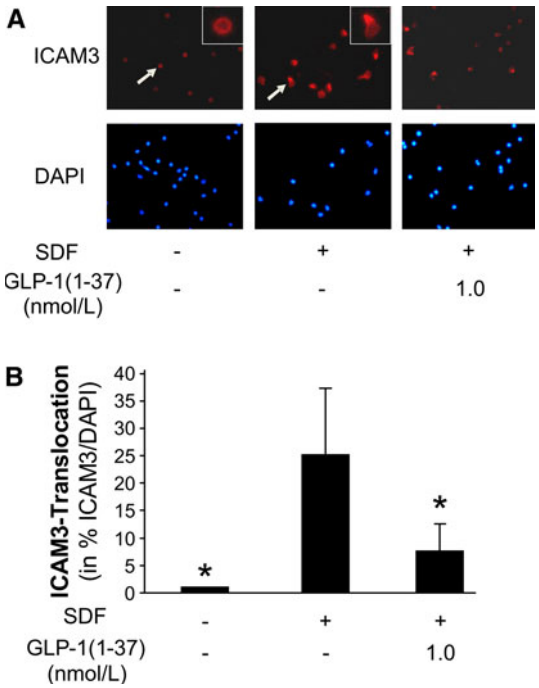

Fig. 4a, b GLP-1(1-37) inhibits SDF-1-induced ICAM3 translocation. **a** CD4-positive lymphocytes were pretreated with GLP-1(1-37) at concentrations indicated before stimulation with SDF-1 for 30 min. ICAM3 translocation was assayed using immunofluorescence staining. *Insets*: high-power view of cells with and without ICAM3 translocation as indicated by *arrows*. **b** Statistical analysis of cells positive for ICAM3 translocation as percentage of DAPI-positive cells. *Bars* represent mean ± SD; $n = 6$; $*p < 0.01$ compared with SDF-1-stimulated cells

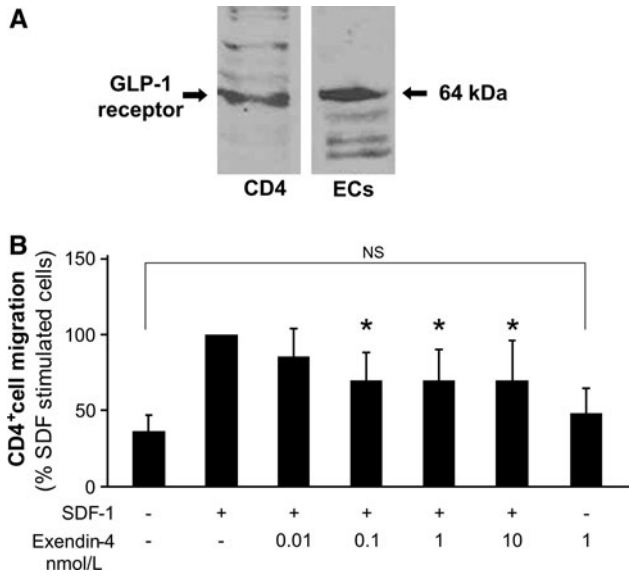

Fig. 5 **a** Human CD4-positive lymphocytes express GLP-1 receptors in vitro. Western blot analysis of isolated human CD4-positive lymphocytes reveals the expression of the GLP-1 receptor protein at the expected size. Lysates from human endothelial cells served as a positive control. Three independent experiments yielded similar results. **b** Exendin-4 inhibits SDF-1-induced migration of human CD4-positive lymphocytes similar to GLP-1(1-37). Human CD4-positive lymphocytes were pretreated with exendin-4 for 30 min at concentrations indicated before migration experiments were performed in a modified Boyden chamber using SDF-1 (100 ng/mL). Data are expressed as percentage of SDF-stimulated cells. *Bars* represent mean ± SD; $n = 12$; $*p < 0.05$ compared with SDF-1-stimulated cells. Exendin-4 did not induce cell migration compared to unstimulated cells

receptor. Western blot analysis of total lysates revealed that isolated human CD4-positive lymphocytes express GLP-1 receptor protein (Fig. 5a) and transfection of cells with GLP-1 receptor siRNA markedly reduced this expression (Fig. 6a). As GLP-1(1-37) is known to be a weak agonist on the GLP-1 receptor, we investigated whether exendin-4, a potent GLP-1 receptor agonist, has similar effects. Pretreatment with exendin-4 inhibited SDF-1-induced migration of human CD4-positive lymphocytes (Fig. 5b).

To investigate whether GLP-1(1-37)'s action on cell migration is dependent on the GLP-1 receptor, we next evaluated the effect of GLP-1(1-37) on SDF-1-induced ICAM3 translocation as a read-out for cell migration in mock and GLP-1 receptor siRNA transfected cells. As shown in Fig. 6b and c, GLP-1(1-37) no longer inhibited SDF-1-stimulated ICAM3 translocation in GLP-1 receptor negative cells compared to mock transfected cells, suggesting that GLP-1(1-37) exhibits its effects on CD4-positive lymphocyte migration and ICAM3 translocation via the GLP-1 receptor.

Discussion

The present study demonstrates that the hormone GLP-1(1-37) inhibits chemokine-induced migration of human CD4-positive lymphocytes. This effect is mediated via the GLP-1 receptor and through a reduction in PI-3 kinase activity with subsequent inhibition of f-actin formation and ICAM3 translocation.

GLP-1(1-37) is mainly secreted from cells in the pancreas and is a less potent GLP-1 receptor agonist than truncated GLP-1(7-36) amide [12, 13]. Recently, GLP-1(1-37) has been shown to convert intestinal epithelial cells into insulin-producing cells [14], thus suggesting a new therapeutic tool for the treatment of diabetes mellitus. Our study extends the current knowledge on GLP-1(1-37) by demonstrating an inhibition of chemokine-induced CD4-positive lymphocyte migration, an effect which could potentially contribute to the modulation of vascular inflammation in diabetic patients. The effect of GLP-1(1-37) on lymphocyte migration is mediated by an early inhibition of PI-3 kinase activity. PI-3 kinase activation is a critical step in chemokine-induced T-cell migration,

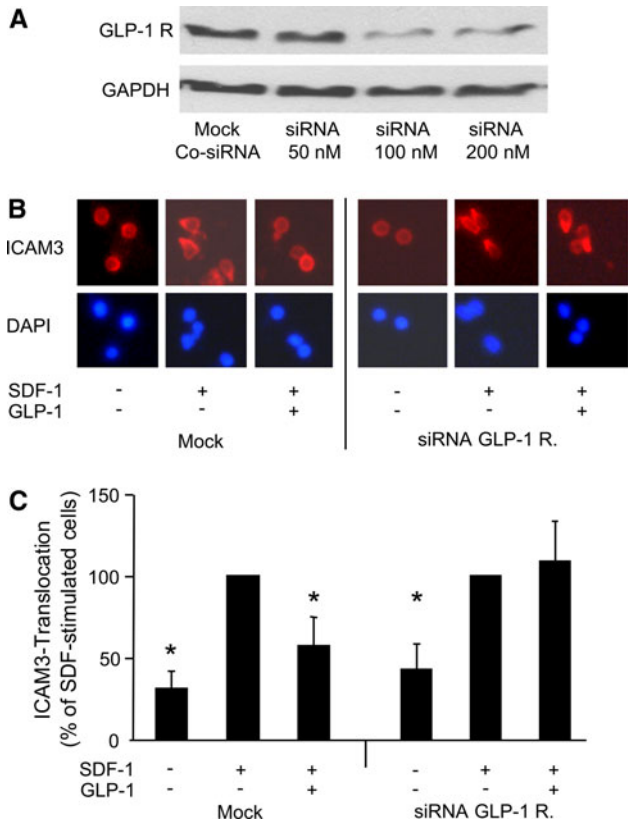

Fig. 6a–c Transfection of CD4-positive lymphocytes with GLP-1 receptor siRNA abolishes the effect of GLP-1(1-37) on SDF-1-induced ICAM3 translocation. **a** Western blot analysis of GLP-1 receptor expression in mock and GLP-1 receptor siRNA-transfected cells (increasing concentrations). **b**, **c**. Mock and GLP-1 receptor siRNA-transfected cells were pretreated with 1 nmol/L GLP-1(1-37) before stimulation with SDF-1. ICAM3 translocation as a read-out for migrating cells was assayed using immunofluorescence staining. **b** Representative pictures are shown. **c** Statistical analysis of cells positive for ICAM3 translocation as percentage of DAPI-positive cells. *Bars* represent mean ± SD as percentage of SDF-1-stimulated cells; $n = 6$; $^*p < 0.05$ compared to SDF-1-stimulated cells

leading to downstream activation of small Rho GTPases. Downstream of these GTPases, the phosphorylation of cofilin is of importance to allow for actin polymerization at the leading edge of migrating cells, while the phosphorylation of MLC contributes to cell contraction at the uropod. Downstream of PI 3-kinase, GLP-1(1-37) leads to a reduction in chemokine-induced phosphorylation of cofilin with a subsequent reduction in f-actin formation. Moreover, GLP-1(1-37) limits the phosphorylation of MLC and inhibits SDF-1-mediated ICAM3 translocation to the uropod of migrating cells, thus counterbalancing two additional steps in cell movement. The effect of GLP-1(1-37) on cell migration does not depend on the chemotactic stimulus since GLP-1(1-37) diminished both SDF-1- and RANTES-induced lymphocyte migration. Moreover, the effects observed are not due to alterations in cell viability or chemokine receptor expression. Finally, the effect observed is not due to unspecific effects of the peptide nor is it caused by endotoxins as shown by the experiments with the scrambled GLP-1(1-37).

Our data demonstrate the expression of the GLP-1 receptor in human CD4-positive lymphocytes, and exendin-4, a known GLP-1 receptor agonist, inhibited chemokine-induced migration of human CD4-positive lymphocytes similar to GLP-1(1-37). In addition, GLP-1(1-37) did not inhibit chemokin-induced ICAM3 translocation in GLP-1 receptor negative human CD4-positive lymphocytes, suggesting that the effect of GLP-1(1-37) on these cells is dependent on the known GLP-1 receptor.

However at this point degradation/cleavage of GLP-1(1-37) in the Petri dish during the time of incubation cannot be entirely excluded in our study. Therefore, a cleavage product of GLP-1(1-37), possibly a truncated form of GLP-1 or GLP-1(9-36), could theoretically be responsible for the inhibition of chemokine-induced migration of human CD4-positive lymphocytes. Future studies are warranted to further elucidate the role of possible cleavage products of GLP-1(1-37) on lymphocyte function.

The GLP-1(1-37) concentrations employed in our study correspond to the concentrations used in previous in vitro experiments [14] and are within the physiological range of GLP-1 plasma concentrations in patients [20]. This raises the possibility that GLP-1(1-37) could act as a physiological modulator of lymphocyte function in vivo and potentially provides a novel tool to influence vascular disease in patients with diabetes. The inhibition of lymphocyte migration shown here may hamper lesion development at a critical step. CD4-positive cells, once recruited into the subendothelial space by chemokines such as SDF-1 or RANTES, differentiate into TH1-cells, thus releasing pro-inflammatory cytokines, which then promote inflammatory activation of other cells in the vessel wall [1]. Therefore, modulating the migration of T lymphocytes into the vasculature may target the inflammatory process in atherogenesis at a nodal point and as such modulate a critical step in lesion development. Future studies are warranted to determine whether such effects of GLP-1(1-37) finally lead to a reduction in lesion size in animal models of arteriosclerosis.

Acknowledgments This work was supported by grants of the Deutsche Forschungsgemeinschaft (DFG-MA2047/4-1; DFG-WA 2145/1-1) to Prof. Dr. Nikolaus Marx and Dr. Daniel Walcher, grants from the Landesforschungsschwerpunkt Baden-Württemberg and the Novartisstiftung für therapeutische Forschung to Prof. Dr. Nikolaus Marx, as well as by a grant from the Karl und Lore Klein-Stiftung to Dr. Mathias Burgmaier. In addition, this project was supported by a grant from the Else-Kröner-Fresenius-Stiftung to Dr. Daniel Walcher. The Department of Internal Medicine II has received an unrestricted research grant from MSD.

Conflict of interest statement None.

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