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Different mechanisms involved in liraglutide and glucagon-like peptide-1 vasodilatation in rat mesenteric small arteries

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Running title: Different effect of GLP-1 and liraglutide on vascular tone

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List of author contributions

Maj Bangshaab contributed to the experimental design, performed and analyzed the experiments in rat and human resistance arteries and wrote a first draft of the manuscript. Khiem Dinh Huynh recruited patients and collected the tissue samples. Alejandro Gutierrez, Jakob Schöllhammer Knudsen, Asbjørn G. Petersen, and Daniel Dias Rufino Arcanjo performed additional experiments in rat mesenteric arteries. Michael Gejl and Jørgen Rungby contributed to the experimental design and the writing. Ulf Simonsen participated in the design and analysis of the experiments and contributed to the writing.

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Abstract

Background and Purpose: Glucagon-like peptide-1 (GLP-1) is an incretin hormone that regulates insulin biosynthesis and secretion in a glucose-dependent manner, and has been reported to induce vasodilatation. Here, the aim was to examine the possible vasorelaxant effect of GLP-1 and the underlying mechanisms.

Experimental Approach: Rat mesenteric arteries (diameter ≈ 200-400 μm) and human subcutaneous arteries were mounted in microvascular myographs for isometric tension recordings. The effect of GLP-1 on vascular responses was examined at normoglycemic conditions and at high glucose.

Key Results: In rat mesenteric arteries and human subcutaneous arteries without branches, physiological concentrations (1-100 nM) of GLP-1(7-36) and liraglutide failed to cause relaxation, or affect contractions evoked by electrical field stimulation. In contrast to GLP-1(7-36), liraglutide induced relaxations antagonized by the GLP-1 receptor antagonist, exendin-(9-39) in branched mesenteric arteries. In contrast to liraglutide, GLP-1 leftward shifted concentration relaxation curves for bradykinin in subcutaneous arteries from patients with peripheral arterial disesase, an effect insensitive to exendin-(9-39). In normoglycemic conditions neither GLP-1 nor liraglutide changed acetylcholine relaxation in rat mesenteric arteries. In arteries exposed to 40 mM glucose, GLP-1, in contrast to liraglutide, potentiated acetylcholine-induced relaxation by a mechanism that was not antagonized by exendin-(9-39). GLP-1 decreased superoxide levels measured with dihydroethidium in rat mesenteric arteries exposed to 40 mM glucose.

Conclusion and Implications: Our findings suggest that a GLP-1 receptor-dependent mechanism is involved in liraglutide relaxation in branched arteries in normoglycemic conditions, while GLP-1 inhibition of vascular superoxide levels contributes to GLP-1

receptor-independent potentiation of endothelium-dependent vasodilatation in hyperglycemia.

Keywords: GLP-1, liraglutide, resistance vessels, high glucose, endothelium dependent relaxation, neuronal inhibition.

Abbreviations:

ACh, acetylcholine; AUC, area under the curve; DPP-4, dipeptidyl peptidase 4; GLP-1, glucagon-like peptide-1; GLP-1R, glucagon-like peptide-1 receptor; EFS, electrical field stimulation; PEG-SOD, polyethylene glycol superoxide dismutase.

Bullet summary

What is already known?

- GLP-1 is an incretin hormone reported to induce vasodilatation and lower blood pressure

What this study adds?

- GLP-1 and the GLP-1 analogue induce vasodilatation by GLP-1R independent and dependent mechanisms

Clinical significance?

- Different GLP-1 analogue effects may help in optimizing prevention of cardiovascular complications in type-2 diabetes

Introduction

Glucagon-like peptide-1 (GLP-1) is a gut-derived incretin hormone secreted in response to nutrient ingestion. GLP-1 has a blood glucose lowering effect by stimulating an increase in insulin biosynthesis and secretion and by inhibiting glucagon secretion in a glucose dependent manner. Moreover, GLP-1 inhibits gastric emptying, augments satiety, and promotes a weight loss (Holst, 2004;Davies *et al.*, 2015). Growing evidence suggests that it may also play a role in the regulation of cardiovascular function (Drucker, 2016). The GLP-1 analogue liraglutide is approved for the treatment of type 2 diabetes mellitus (T2D) and obesity (Ussher & Drucker, 2014;EMA, 2017) and has been found to reduce blood pressure and the risk of cardiovascular events (Fonseca *et al.*, 2014;Gejl *et al.*, 2015;Marso *et al.*, 2016;Starup-Linde *et al*., 2014). GLP-1 and liraglutide bind to the GLP-1 receptor (GLP-1R), a G-protein-coupled receptor (Ussher & Drucker, 2014; Alexander *et al.*, 2017). In the cardiovascular system, the GLP-1R has been found to be expressed in smooth muscle cells, the sinoatrial node (Pyke *et al.*, 2014), the myocardium and microvascular endothelium (Ban *et al*., 2008;Bhashyam *et al.*, 2010). However, some papers have questioned data obtained using standard antisera to detect the GLP-1R (Pyke & Knudsen, 2013;Panjwani *et al.*, 2012).

GLP-1R agonists have been found to induce vasodilatation, increase blood flow, and to improve vascular endothelial function (Drucker, 2016;Gejl *et al.*, 2012), but the results of studies in isolated arteries are conflicting. Generally, it has been assumed that the vasodilatory actions of GLP-1 is GLP-1R-dependent, but some studies proposed vascular effects independent of the GLP-1R (Ban *et al.*, 2008). It has also been questioned whether the cleaved metabolite, GLP-1(9-36), of native GLP-1(7-36) plays a role (Bayram *et al.*, 2014;Ban *et al.*, 2008). Several pathways have been suggested to mediate the vascular effects of GLP-1R agonists. These includes direct effect on the smooth muscle cells, endothelium-dependent release of nitric oxide (NO) (Nystrom *et al.*, 2005), and pathways involving an

increase in cyclic GMP or cyclic AMP as well as protection against endothelial dysfunction (Ban *et al.*, 2008;Green *et al.*, 2008;Bayram *et al.*, 2014;Koska *et al.*, 2015;Salheen *et al.*, 2015). However, an indirect vasoconstricting effect of GLP-1 causing inhibition of relaxations induced by vascular endothelial growth factor (VEGF) has also been described in mesenteric arteries and was associated with a decrease in endothelial cell calcium (Egholm et al., 2016). Therefore, the effect on vascular tone of GLP-1 is unclear, and a possible effect of GLP-1 on the peripheral autonomic nervous system has not been examined despite the fact that several other gut-derived peptides regulate the release of noradrenaline in the vasculature (Gradin *et al.*, 2006). Moreover, hyperglycemia generates reactive oxygen species suggested to impair endothelium dependent relaxation, and growing evidence reveals that GLP-1 receptor stimulation may reduce ROS (Salheen *et al*., 2015;Steven *et al.*, 2017), and hence improve endothelial function (Salheen *et al.*, 2015).

In the present study, we aimed to examine the pre-junctional and post-junctional vasorelaxant effects of native GLP-1(7-36), liraglutide and GLP-1(9-36) in rat and human resistance vessels, to elucidate the signaling pathways involved in GLP-1-mediated regulation of vascular tone. We hypothesized that GLP-1R stimulation regulates vascular tone either by a direct impact on signaling pathways determining the contractile level of the smooth muscle cells, by an endothelium-dependent pathway, or through inhibition of noradrenaline release from nerves in the vascular wall during conditions with increased sympathetic nerve activity. Moreover, we aimed to investigate whether GLP-1 by inhibition of superoxide may improve acetylcholine relaxations in rat mesenteric arteries exposed to hyperglycemic conditions.

Materials and methods

Tissue preparations

All animal experiments were conducted in accordance with the Danish legislation of animal use for scientific procedures and approved by the Danish Experimental Animal Inspectorate (permission 2014-15-2934-01059) according to the ARRIVE guidelines (McGrath & Lilley, 2015). Adult male Wistar rats (10-12 weeks old) of the Hannover strain (RGD Cat# 1566433, RRID:RGD_1566433) and weighing 300-350 g were obtained either at Taconic, Ry, Denmark or from Janvier Laboratories, France. The rats were housed in cages (Techniplast, 800 cm^2) filled with aspen woodchips (Tapvei, Datesand, Manchester, UK) and nesting material (Soft Paper Wool/LBS, UK) as bedding and under constant climatic conditions (20°C, 60 % humidity, 12-h:12-h light-dark cycle). The animals got food (Altromin 1324, Brogaarden ApS, DK) and water ad libitum.

The animals were selected randomly and, wherever possible, observations were made without knowledge of the treatments administered. The rats were killed by a blow to the head followed by exsanguination. The mesenteric bed was removed, and it was immediately placed in cold physiological salt solution (4°C) of the following composition (mM): NaCl 119, KCl 4.7, KH_2PO_4 1.18, $MgSO_4$ 1.17, $NaHCO_3$ 25, $CaCl_2$ 1.6, EDTA 0.026 and glucose 5.5. The solution was gassed with 5% CO_2 in air to maintain the pH at 7.4. Third order mesenteric arteries (internal diameter ≈ 200-300 µm) were dissected without branches (straight segments) or with at least one major branch (branched vessels).

Human arteries were obtained from the Department of Vascular Surgery, Aalborg University Hospital. Subcutaneous tissue samples were obtained from 23 patients undergoing vascular surgery, and they were placed in cold physiological salt solution immediately after extraction. Subcutaneous arteries with internal diameters ≈ 200-400 µm were dissected. The study was approved by the Regional Ethics Committee, Central Denmark (permission

number: 1-10-72-208-15), and conducted in accordance with the principles of Helsinki Declaration II for medical research. All participants gave informed consent prior to participation. To be included in the study, the patients had to be, 1) adult competent persons, 2) Caucasians, 3) diagnosed with peripheral arterial disease or ischemic heart disease. Both diabetic and non-diabetic patients were included. The patients were excluded, if they were currently treated with GLP-1 analogues.

Isometric tension recordings

Arterial segments (approximately 2 mm long) were mounted on two wires (40 µm in diameter) in microvascular myographs (Danish Myotechnology, Aarhus, Denmark) for isometric tension recordings. The vessels equilibrated in oxygenated (5% CO_2 in air) physiological salt solution (pH 7.4) heated to 37°C. To determine the lumen diameter that the artery would have had in vivo when relaxed and under a transmural pressure of 100 mmHg, the vessels were stretch following a standardized procedure. Arteries were set to 90% of this diameter, which is the optimal vessel circumference for maximal force development (Mulvany & Halpern, 1977). To test tissue viability, arterial segments were subjected to an isotonic physiological salt solution containing a high concentration of potassium (123 mM) or noradrenalin (1-10 µM) resulting in maximal contraction of the vessels. To test endothelial function, the vessels were contracted with phenylephrine or noradrenalin, and then stimulated with acetylcholine (10 µM) in rat arteries, and with bradykinin (30 nM) in human arteries. Only rat mesenteric arteries relaxing more than 75% to acetylcholine and human subcutaneous arteries relaxing more than 50 % to bradykinin were included in the study.

The possible direct effect of GLP-1 and analogues in rat and human resistance arteries was examined by adding increasing cumulative concentrations of GLP-1(7-36), liraglutide or GLP-1(9-36) (0.1 pM – 100 nM) to vessels contracted with phenylephrine (5 µM – 10 µM). To address the potential development of tachyphylaxis a single physiological concentration

of GLP-1(7-36) (1 nM) was added in a series of experiments. To investigate whether GLP-1 was degraded, preparations were incubated with a dipeptidyl peptidase (DPP-4) inhibitor, linagliptin (10^{-6} M) before adding GLP-1. All experiments were compared to a phenylephrine-contracted parallel running time control vessel.

To consider whether the vaso-regulatory effect of GLP-1 is endothelium-dependent, we studied if incubation with therapeutic concentrations (10 nM) of GLP-1(7-36), liraglutide and GLP-1(9-36) for 20 min. before adding increasing cumulative concentrations of acetylcholine (0,1 nM – 10 µM), affected the acetylcholine induced relaxation in rat mesenteric arteries contracted with phenylephrine (10 µM). The experiments were performed at a low glucose concentration of 5.5 mM and at a very high glucose concentration as a model of hyperglycemia. In the hyperglycemia condition the vessels were incubated for at least two hours with 40 mM of glucose before running the protocol, as described by Salheen *et al.* (2015). The very high concentration of glucose was based on findings in previous studies, demonstrating that incubation with a similar high glucose concentration (44 mM) for a short time (4-6 hours) impaired endothelium-dependent relaxation in isolated vessels (Tesfamariam *et al.*, 1991;Qian *et al.*, 2006). The responses were examined during co-incubation with the GLP-1R antagonist exendin-(9-39) (100 nM). Similar experiments were performed in human subcutaneous arteries**.** Concentration-response curves for another endothelium-dependent relaxant, bradykinin (0.1 pM – 100 nM) was obtained in human arteries contracted with phenylephrine (5 µM) and preincubated with therapeutic concentrations (10 nM) of GLP-1(7-36) and liraglutide. Furthermore, human arteries were incubated with a combination of GLP-1(7-36) and exendin-(9-39) (100 nM) to examine whether the effect of GLP-1 is mediated through GLP-1 receptors. In all the experiments, equivalent solvent concentrations were added to a parallel running time control (vehicle).

To investigate the role of superoxide in the inhibition of acetylcholine relaxations in high glucose, mesenteric arteries exposed to 40 mM for 2h of glucose were incubated with vehicle or the superoxide dismutase scavenger, tempol (10^{-4} M), and a concentration-response curve was constructed for acetylcholine. To clarify whether the GLP-1-receptor independent effect of GLP-1 on acetylcholine relaxation in high glucose is due to an antioxidant effect of GLP-1, rat mesenteric arteries were incubated with indomethacin (10^{-6} M) and the superoxide generator pyrogallol (30 µM) for 30 min, phenylephrine was added, and the preparations were incubated with either vehicle, GLP-1 (10^{-8} M), liraglutide (10^{-8} M), tempol (10^{-4} M), or linagliptin (10^{-6} M). Finally, concentration-response curves were constructed for acetylcholine.

Detection of vascular O_2^-

The vascular O_2^- was detected using the oxidative fluorescent dye dihydroethidium (Cat# D1137, Invitrogen, Thermo Fisher Scientific, Denmark) (Symons *et al.*, 2006;Christensen *et al.*, 2007). Freshly dissected mesenteric arteries were placed in PSS in Ependorf vials at 37 ^{O}C. They were kept in normoglycemic conditions (5.5 mM glucose) or in high glucose (40 mM) for 2½h, and those with high glucose were during the last 30 min incubated with vehicle, polyethylene glycol superoxide dismutase (PEG-SOD, 900 U ml^{-1}), GLP-1 (10^{-8} M), liraglutide (10^{-8} M), or linagliptin (10^{-6} M). All segments were incubated with dihydroethidium (4 µmol/l) or with phosphate-buffered saline (PBS, vehicle/time control) at 37°C in light-protected vials, rinsed once with 400 µl to PBS to remove un-oxidized dihydroethidium, and mounted on slides with the mounting medium Vectashield (Vector Laboratories, Inc. Burlingame, California). O_2^- production was estimated using fluorescence microscopy (Leica, Mannheim, Germany) with an ethidium bromide excitation, 488 nm; Ethidium bromide emission, 580- to 630-nm band-pass filter. Fluorescence measurements were performed, and the vascular area measured calculated on basis of white-light pictures.

Identical settings were used for all vascular segments and pictures. Relative fluorescence intensity (RFI) was measured using the image analysis software (Fiji, RRID:SCR_002285) (Schindelin *et al.*, 2012). The data are reported as PEG-SOD sensitive fluorescence.

Electrical field stimulation

Electrical field stimulation (EFS) was performed as previously described (Simonsen *et al.*, 2008). Platinum electrodes (2 x 2 mm) secured in plastic mounting heads were placed on either side of the vessel approximately 1 mm from the vessel wall. The electrodes were connected to an electrical stimulator with a constant current output (CS200, Danish Myotechnology, Aarhus, Denmark). EFS (16 Hz, 0.3 ms pulses, 10 s trains, 40 mA) was applied every 5 min. Increasing cumulative concentrations of GLP-1(7-36) and liraglutide (0,1 nM – 300 nM) were added 3 min before each stimulation. The contractile responses were expressed relative to an average of two initial control stimulations and compared to a parallel running time control. To obtain reproducible contractions, propranolol (1 μM), a β-adrenoceptor blocker, and cocaine (1 μM), an inhibitor of neuronal noradrenalin reuptake, were added. The vessels were also stimulated in the presence of tetrodotoxin, a blocker of the voltage-gated sodium channels in nerve cell membranes.

Data calculations and statistical analysis

The data and statistical analysis comply with the recommendations on experimental design and analysis in pharmacology (Curtis *et al.*, 2015). In view of previous experience, each group had to contain preparations from minimum 5-6 different subjects. Relaxation and contractile responses in the arteries were measured as the force ΔF (mN) developed by the artery, and illustrated the changes in active wall tension. Relaxation in contracted arteries was presented as percentage of the contraction level just before adding the relaxing agents. Differences in concentration-response relationships between treatments were analyzed using

two-way analysis of variance (ANOVA) followed by Bonferroni correction. The fluorescence results for vascular superoxide were compared using one-way ANOVA followed by Turkey multiple comparisons test. Comparison of -logEC_{50} values was analyzed with a t test. Results were considered statistically significant at $p<0.05$. GraphPad Prism 7 (GraphPad Prism, RRID:SCR_002798, Inc., San Diego, CA) was used for the statistical analysis.

Reagents

Acetylcholine, bradykinin, linagliptin, noradrenalin hydrochloride, phenylephrine, propranolol, cocaine, tempol and Glucagon-Like Peptide 1 amide Fragment 7-36 (human) were all purchased from Sigma-Aldrich (St Louis, MO, USA). GLP-1(7-36) amide (human, rat), GLP-1(9-36) amide, Exendin-3(9-39) amide, were from Tocris bioscience (Bristol, UK). Tetrodotoxin from BioNordika (Herlev, DK). All chemicals were dissolved in distilled water except for Glucagon-Like Peptide 1(7-36) human (1 % acetic acid). Victoza injection liquid (liraglutide) from Novo Nordisk (Copenhagen, DK) was diluted in bubbled PSS without Ca^{++} at 37 °C.

Nomenclature of Targets and Ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Harding *et al.*, 2018), and are permanently archived in the Concise Guide to PHARMACOLOGY 2017/18 (Alexander *et al.*, 2017).

Results

Direct effect of GLP-1R agonists on smooth muscle cell relaxation

In straight rat and human resistance arteries contracted with phenylephrine, native GLP-1 failed to cause relaxation. When adding a single physiological concentration of GLP-1 (1 nM) to the contracted vessels, the observed response was similar to the loss of tone in the parallel running time control (Figure 1A-B, supplementary Figure S1). Concentration-response curves in both rat mesenteric arteries and human subcutaneous arteries showed no or only a very modest effect, as the observed GLP-1 mediated relaxation was close to 0% (Figure 1C-D). Incubation with a DPP-4 inhibitor, linagliptin (10^{-6} M) did not cause relaxation or increase the response to GLP-1 (Figure S1).

Likewise, no vasorelaxant effect was found for native GLP-1 or GLP-1(9-36) in branched mesenteric arteries (Figure 2A-B). Whereas liraglutide induced significant relaxation compared with vehicle, to approximately 40 % of maximal relaxation as demonstrated on the concentration-response curve in branched mesenteric arteries (Figure 2C). This effect was antagonized by adding of the GLP-1R antagonist exendin(9-39) (Figure 2D).

Effect of GLP-1R agonists on the EFS evoked contractions

EFS was performed to examine the potential influence of GLP-1 on the sympathetic nerves in the vascular wall. Representative traces from the EFS experiments illustrate the lack of inhibitory effect of GLP-1 and liraglutide on the EFS responses (Figure 3A, Figure S2), indicating that GLP-1 and liraglutide have no effect on the neurotransmitter-release from the nerves in the vascular wall. GLP-1 and liraglutide did not have any significant effect on either the amplitude of the contractions or the area under the contraction curve (AUC, Figure 3B-C). Tetrodotoxin, a blocker of the voltage-gated sodium channels in nerve cell membranes,

inhibited the EFS evoked contractions. This ensured that the contractions examined here were due to stimulation of the nerves and not the smooth muscle cells.

Effect of GLP-1 and liraglutide on endothelium-dependent relaxation in human subcutaneous arteries

In human subcutaneous vessels from patients with peripheral arterial disease (Table 1), GLP-1 (10^{-8} M) leftward shifted concentration relaxation curves for the endothelium-dependent vasodilator, bradykinin (Figure 4A). Incubation with liraglutide (10^{-8} M) failed to change the concentration-response curves for bradykinin in human subcutaneous arteries (Figure 4B).Vessels were obtained from both patients diagnosed without (non-diabetics) (n=6) and diabetic patients (n=4). GLP-1 also leftward shifted the concentration-response curves for bradykinin in subcutaneous vessels from non-diabetic patients (n=6), while the effect was not apparent in subcutaneous arteries from patients with diabetes (n=4; Supplementary Figure S3). In a pooled sample of arteries from patients with and without diabetes, GLP-1 still leftward shifted the concentration-response curves for bradykinin in the presence of the GLP-1R antagonist exendin(9-39) (n=5, Figure 4C).

Effect of GLP-1 and liraglutide on endothelium-dependent relaxation in rat mesenteric small arteries

At a normoglycemic condition of 5.5 mM glucose, GLP-1 did not change the relaxation to acetylcholine in rat mesenteric arteries (Figure S4). In the first set of experiments with the model of hyperglycemia, incubation of rat mesenteric vessels with 40 mM of glucose significantly impaired the relaxant response to acetylcholine (Figure 5A). Based on these findings and to ensure, that only vessels that had actually been affected by the hyperglycemic condition were included in the GLP-1 study, experiments with a 40 mM glucose control vessel that relaxed more than 40 % at 30 nM acetylcholine was excluded (n = 6). GLP-1

significantly improved the relaxation to increasing concentrations of acetylcholine at a high glucose concentration (Figure 5B). The response to acetylcholine at high glucose in the presence of GLP-1 was similar to the response to acetylcholine at a low glucose concentration. Exendin(9-39) did not block this effect (Figure 5C). The GLP-1 potentiation of acetylcholine-induced relaxation was examined at GLP-1 concentrations of 0.1-10 nM, though only 10 nM GLP-1 significantly improved acetylcholine potency (Figure S4). The GLP-1 analogue, liraglutide, and the metabolite of GLP-1, GLP-1(9-36) failed to change acetylcholine-induced relaxations (Figure 5D and 5E). Incubation with the superoxide scavenger, tempol (10^{-4} M) leftward shifted concentration-response curves for acetylcholine in mesenteric arteries exposed to high glucose (Figure 5F).

Effect of GLP-1 and liraglutide on vascular superoxide levels

To investigate the role of superoxide in the effects of GLP-1, rat mesenteric arteries were exposed to the superoxide generator pyrogallol in the absence and the presence of GLP-1 and liraglutide. In the presence of pyrogallol, concentration-response curves for acetylcholine were leftward shifted after incubation with GLP-1 (Figure 6A), while the presence of liraglutide failed to change the curves for acetylcholine in arteries exposed to pyrogallol (Figure 6B).

To directly measure vascular superoxide levels, the preparations were stained with DHE (Figure 7A-F). The changes in fluorescence was expressed relative to the effect of the superoxide scavenger, PEG-SOD (Figure 7G). Incubation with high glucose (40 mM) markedly increased the vascular superoxide levels compared to vessels kept at 5 mM glucose. GLP-1 decreased the superoxide levels in vascular preparations exposed to high glucose, while the effect was less pronounced for liraglutide and linagliptin (Figure 7G).

Discussion

The present study demonstrates that liraglutide induces vasorelaxation in branched rat mesenteric arteries, whereas no direct vasodilating effect and no effect on the sympathetic nerves in the vascular wall of native GLP-1, GLP-1(9-36) or liraglutide was found in resistance arteries without branches. In contrast to liraglutide, GLP-1 leftward shifted concentration relaxation curves for bradykinin in subcutaneous arteries from patients with peripheral arterial disesase, an effect insensitive to exendin(9-39). This study also suggests that, at a high glucose concentration, native GLP-1, by a GLP-1 receptor independent mechanism, may potentiate acetylcholine relaxation in small rat mesenteric arteries by decreasing vascular superoxide levels.

The direct GLP-1 effect

Previous studies have suggested a direct vasodilating effect of GLP-1 in isolated mesenteric arteries from rodents and of GLP-1R agonists in human subcutaneous arteries, as an explanation of the blood pressure lowering effect observed in patients treated with GLP-1R agonists (Ban *et al.*, 2008;Bayram *et al.*, 2014;Koska *et al.*, 2015). These studies show an effect at GLP-1R of agonist concentrations varying from 3 pM to 3 nM. We did not find any direct vasodilating effect in human or rat resistance arteries without branches at similar GLP-1 concentrations. In our experiments, GLP-1 failed to cause a relaxing response that was any different from the changes in artery tone in our time controls. Neither did we find any vasoconstricting effect of GLP-1 in mesenteric arteries as has been suggested in a previous in vivo study in rats (Gardiner *et al.*, 2006). None of the studies that found a direct vasodilating effect of GLP-1, describe or show a time control response. Therefore, it is unclear if they have actually considered the changes in artery tone due to time, as an explanation of the observed GLP-1 induced vasodilatation.

Contrary to the findings in vessels without branches, we did observe a direct vasorelaxant effect of liraglutide in branched rat mesenteric arteries that was inhibited by the GLP-1R antagonist, exendin(9-39). This finding suggests a GLP-1R-dependent and consequently exendin(9-39)-sensitive mechanism, consistent with the fact that liraglutide is a selective GLP-1R agonist. A more pronounced expression of GLP-1Rs at branching points in the vascular bed may explain this effect. GLP-1Rs have been found to be expressed in small arteries of the intestine, co-localized with smooth muscle α-actin (Richards *et al*., 2014), supporting the findings of a direct effect of liraglutide in mesenteric branched resistance arteries. Whether the GLP-1R expression is concentrated around branching points, remains to be examined in future studies. Both native GLP-1(7-36) and the metabolite GLP-1(9-36) did not evoke a similar response in branched rat mesenteric arteries. The concentrations of GLP-1 and analogues thereof used in these studies (single adding: 1nM, concentration-response: 0.1 pM - 100 nM) are comparable to plasma concentrations measured after infusion of therapeutic doses of GLP-1 in patients (Toft-Nielsen *et al.*, 1999;Edwards *et al.*, 1998), ensuring translational results. We therefore suggest that liraglutide induces vasorelaxation in branched mesenteric arteries, whereas GLP-1 and analogues has no direct vasodilating effect in isolated rat and human small resistance arteries without branches.

GLP-1 effects on the vascular nerves

GLP-1R agonists have been suggested to increase heart rate by activation of the sympathetic nervous system (Smits *et al*., 2016), and therefore, GLP-1R agonists may also regulate vascular tone by interaction with the nerve endings in the vascular wall similar to suggested effects of other gut-derived peptides (Gradin *et al.*, 2006). This has to our knowledge, not been examined before. EFS is a method that enable us to activate vascular sympathetic nerve endings in a single isolated vessel. In our experiments, GLP-1 and liraglutide did not reduce the vascular contractions due to EFS and evoked by neurotransmitter release from vascular

sympathetic nerves in rat mesenteric arteries. Neither did they increase the responses, suggesting that GLP-1 and liraglutide have no effects on the vascular sympathetic nervous activity in rat mesenteric arteries.

GLP-1 effect on endothelium-dependent relaxation

A possible GLP-1 effect on endothelium-dependent relaxation at normoglycemic conditions was examined in both rat mesenteric and subcutaneous arteries from patients with arterial disease. Previous studies in patients have shown apparently conflicting results about a possible GLP-1 effect on vascular endothelial function. In contrast to healthy subjects, infusion of GLP-1 increased forearm blood flow in patients with T2D (Nystrom et al., 2004), and the GLP-1R agonist exenatide potentiated acetylcholine vasodilatation in adipose tissue arterioles (Koska et al., 2010). Others found no effect of the GLP-1R agonist liraglutide on endothelium-dependent vasodilatation in T2D patients (Nandy et al., 2014). As the human arteries used in this study were obtained from patients with arterial disease, they may have some degree of endothelial dysfunction. In contrast to liraglutide, GLP-1 induced a small leftward shift of the concentration-response curves for bradykinin in the human subcutaneous arteries. Previous studies have shown that exendin-4 potentiates acetylcholine vasodilatation in human adipose arterioles by a GLP-1 receptor-dependent mechanism (Koska et al., 2010). However, in the present study the effect of GLP-1 on bradykinin relaxation persisted in the presence of exendin(9-39) suggesting the effect is GLP-1R independent. Taken together our findings suggest that different mechanism are involved in the effect of, respectively, GLP-1 and GLP-1 analogues on endothelial function in human arteries.

As GLP-1 did not seem to have any effects at normoglycemic conditions in rat mesenteric arteries, it was interesting to examine if a hyperglycemic condition comparable to the state of hyperglycemia in diabetic patients is required for GLP-1 to mediate its actions. Previous studies found that the GLP-1R agonist exendin-4 improved endothelium-dependent

relaxation in vessel exposed to high glucose (Salheen *et al.*, 2015;Koska *et al.*, 2015). We found that exposure of rat mesenteric arteries to high glucose impaired relaxation to acetylcholine, and that native GLP-1 almost reversed this high glucose induced damage. As acetylcholine relaxation is dependent on endothelial function our results suggest that GLP-1 improves endothelial function in vessels that have been exposed to hyperglycemia, consistent with the findings by Salheen et al. (2015) and Koska et al. (2015). A GLP-1 concentration of 10 nM was required to significantly increase the potency of acetylcholine (Figure S4), a concentration similar to the plasma concentrations measured after subcutaneous injection of therapeutic concentrations of liraglutide (Danne *et al.*, 2017;Damholt *et al.*, 2006). Liraglutide or the cleaved metabolite of GLP-1, GLP-1(9-36), did not reproduce the effect of GLP-1. This can be due to differences in chemical structure of GLP-1 and liraglutide (Lund *et al.*, 2014). Nevertheless, we found an effect of GLP-1 on hyperglycemia-induced impairment of endothelium-dependent relaxation, but as we were not able to show a similar effect of liraglutide, it is uncertain if this is the major mechanism resulting in the lowering of blood pressure in diabetic patients treated with GLP-1 analogues. Furthermore, it is noteworthy that GLP-1 plasma levels have been found to be associated with blood pressure in normoglycemic young healthy adults as well (Krisai *et al.*, 2015), demonstrating that there may be other GLP-1 effects of importance that impacts blood pressure.

Exendin(9-39) did not attenuate the effect of GLP-1 suggesting that the effect is independent of the GLP-1R. This assumption is conflicting with the results by Salheen et al. (2015), indicating that further studies are needed to confirm the possible GLP-1R dependence- or independency. However, previous investigations suggested that exendin-4 and the DPP-4 inhibitor, linagliptin potentiate acetylcholine relaxations in rat mesenteric arteries exposed to high glucose by a mechanism decreasing vascular superoxide levels (Salheen *et al.*, 2015). It has also been shown that native GLP-1 decreases monocyte-derived

oxidative stress (Steven *et al.*, 2016). In the present study, a superoxide dismutase mimetic, tempol, leftward shifted concentration-response curves for acetylcholine in arteries exposed to high glucose suggesting vascular superoxide levels play a role in high glucose impairment of acetylcholine relaxation. Moreover, we found that GLP-1 prevented impairment of acetylcholine relaxation by the superoxide generator pyrogallol, while this was not the case for liraglutide. Finally, measurements of vascular superoxide levels by use of dihydroethium revealed that GLP-1 markedly decreased the levels. Our results suggest that one vascular action of therapeutic concentrations of native GLP-1, but not of liraglutide or GLP-1(9-36), is that it improves endothelium-dependent relaxation at a high glucose concentration, possibly by a GLP-1R-independent pathway involving inhibition of vascular superoxide levels.

Conclusions

In summary, in normoglycemic conditions a GLP-1R-dependent mechanism is involved in liraglutide relaxation in branched arteries, while GLP-1 and analogues at therapeutic concentrations have no direct vasodilating effect on the smooth muscle cells in arterial segments without branches and no pre-junctional effect on the neurotransmitter release from the sympathetic vascular nerves. We found that inhibition of vascular superoxide levels contributes to the GLP-1 receptor-independent potentiation of endothelium-dependent vasodilatation in hyperglycemic conditions. The latter mechanism may play a role in GLP-1 induced leftward shift of concentration relaxation curves for bradykinin in subcutaneous arteries from patients with peripheral arterial disease. These studies suggest that different mechanisms are involved in GLP-1 and liraglutide induced vasodilatation in small arteries, and may contribute to the blood pressure lowering effect of GLP-1 analogues previously observed in treatment of type 2 diabetic patients.

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Conflict of interests

The authors have no conflicts of interest to declare.

Declaration of transparency and scientific rigour

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research as stated in the BJP guidelines for Design & Analysis, and Animal Experimentation, and as recommended by funding agencies, publishers and other organizations engaged with supporting research.

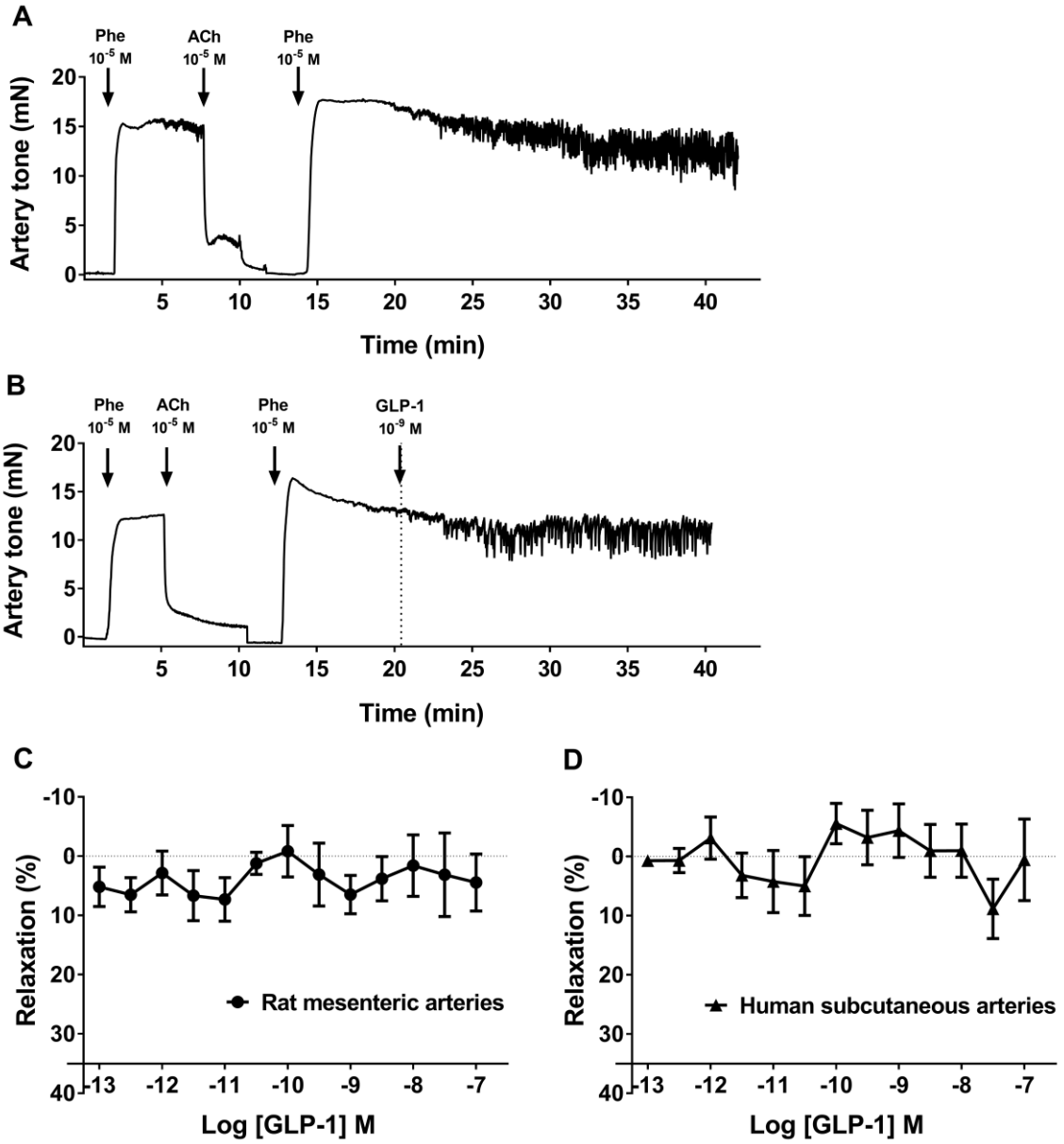

Figure 1. Lack of vasodilatation to native GLP-1 in rat mesenteric and human subcutaneous arteries without branches. (A, B) Representative original traces from the myograph experiments in rat mesenteric arterial segments. Both vessels relax to a sufficient level when adding acetylcholine. (A) Time control contracted with phenylephrine. (B) Vessel contracted with phenylephrine and exposed to a single physiological concentration of GLP-1. Concentration-response curves for GLP-1 in (C) rat mesenteric arteries (n=7) and (D) human subcutaneous arteries (n=9). The results are means ± s.e.mean.

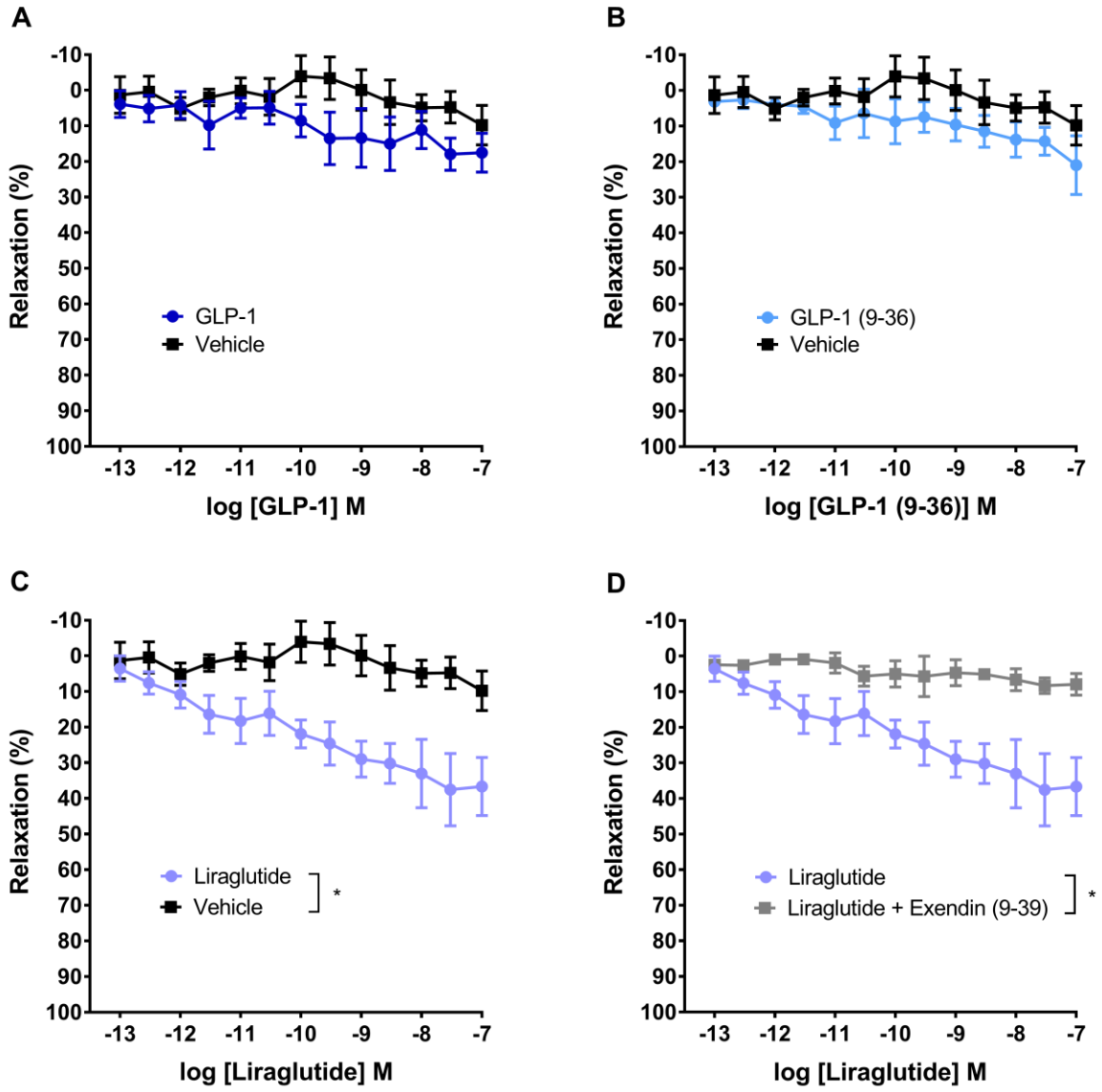

Figure 2. Vasodilatation induced by liraglutide in rat mesenteric arteries with branches. Concentration-response curves for (A) native GLP-1 (n=6) and vehicle (n=6), (B) GLP-1(9-36) (n=6) and vehicle (n=6), (C) liraglutide (n=6) and vehicle (n=6), (D) liraglutide (n=6) and liraglutide plus exendin(9-39) (n= 6). The results are means ± s.e.mean. 2-way ANOVA, *P<0.05.

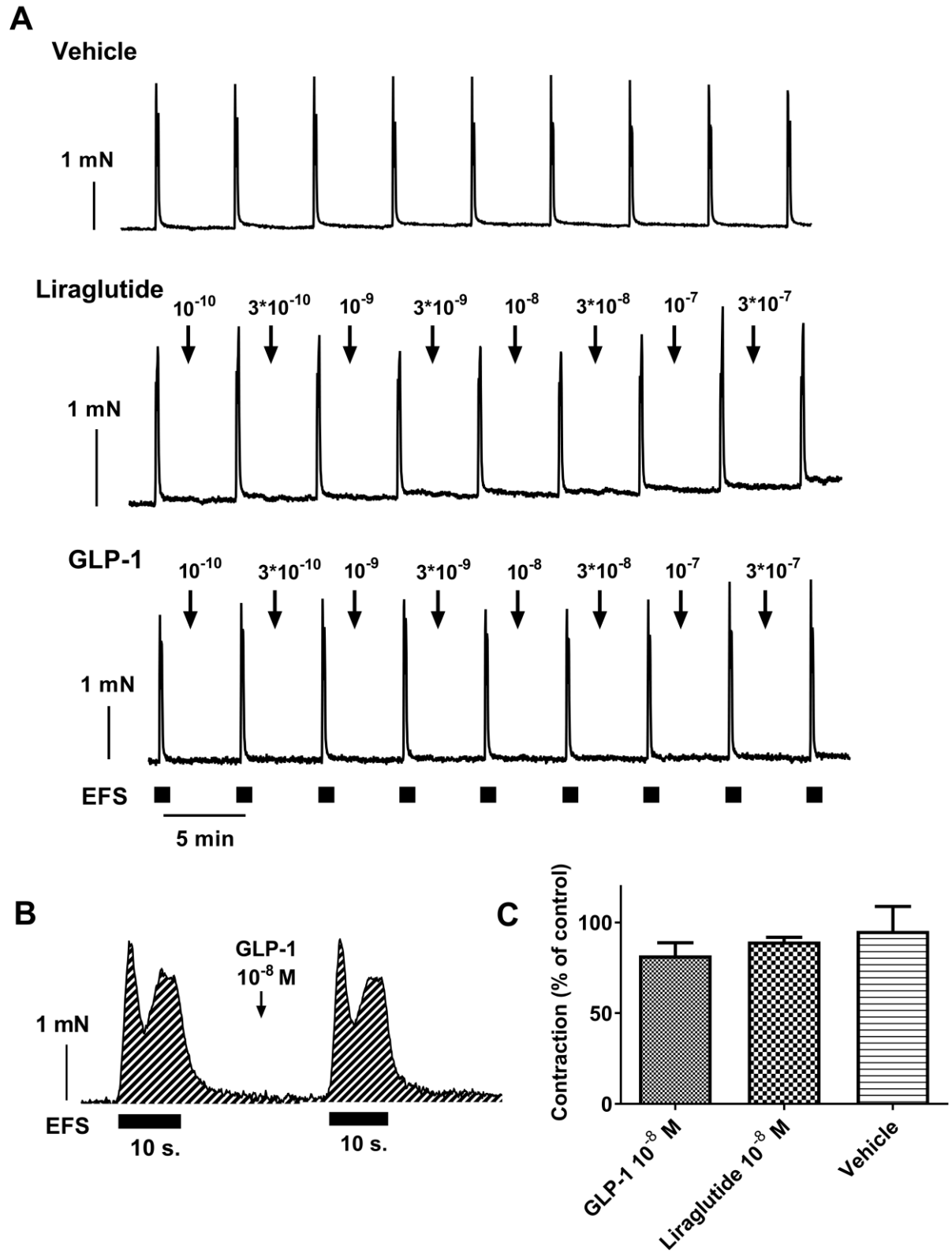

Figure 3. Lack of effect of native GLP-1 and liraglutide on neurogenic contractions in rat mesenteric arteries. (A) Representative original traces showing contractions induced by

electrical field stimulation in the absence and the presence of increasing concentrations (M) of native GLP-1 and liraglutide. Squares mark stimulation intervals (10 s). The traces are each representative of 5-6 different experiments. (B) Enlargement of the responses showing area under the response curve (AUC) at one control stimulation and after adding native GLP-1 (10^{-8} M), (C) Average AUC for the contractions in experiments where vehicle (n=6), GLP-1 (n=6), or liraglutide (n=5) was added. AUC of the contractions are presented as percentage of an initial control stimulation. Data are presented as means ± s.e.mean. Unpaired t-test showed no significant difference between treatments.

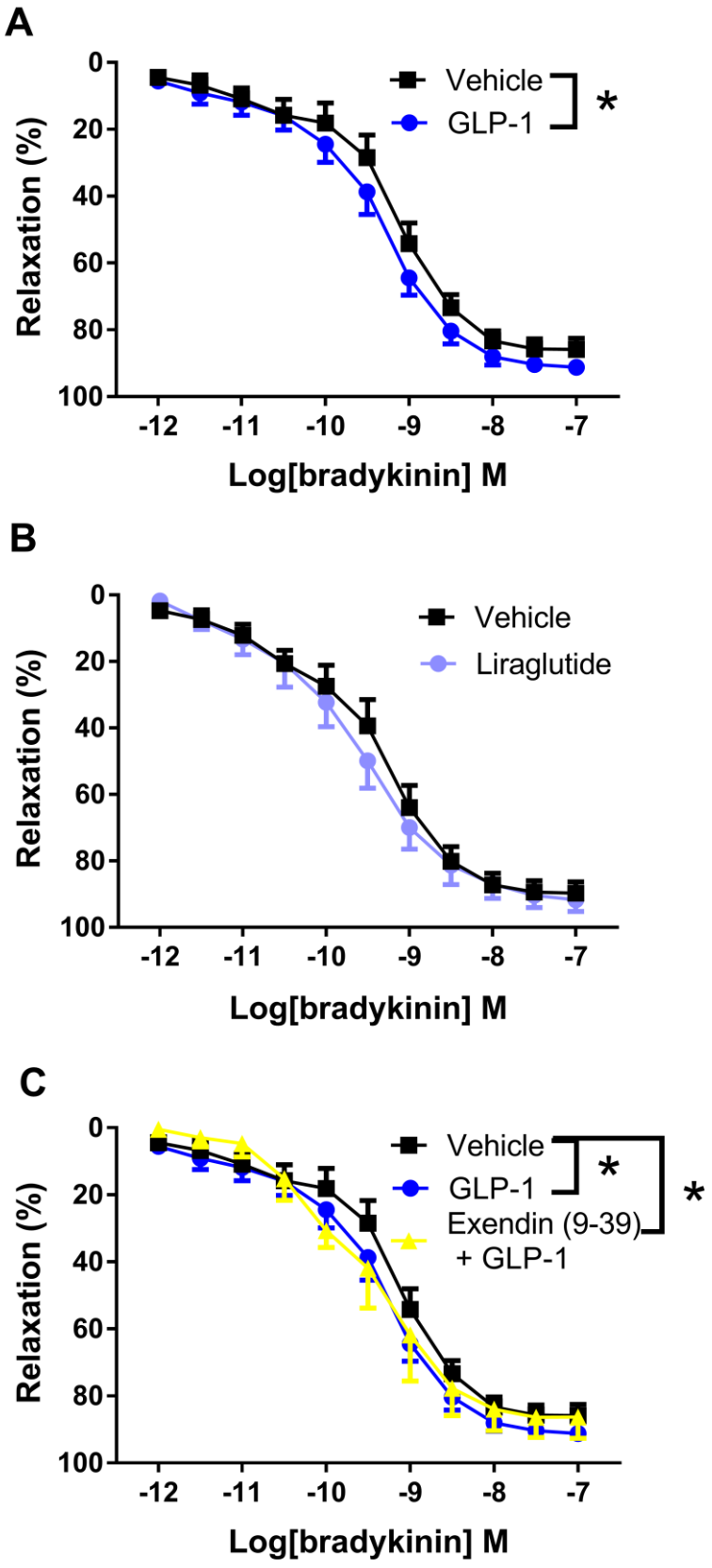

Figure 4: Bradykinin induced relaxation in human subcutaneous arteries. (A) Average concentration-response curves for bradykinin in the absence (n=10) and in the presence of GLP-1 (10^{-8} M, n=10). (B) Concentration-response curves for bradykinin in the absence (n=10) and in the presence of liraglutide (10^{-8} M, n=10). (C) Average concentration-response curves for bradykinin in the absence and in the presence of GLP-1 (10^{-8} M), and GLP-1 plus the GLP-1 receptor antagonist, exendin(9-39) (n=5). The results are means ± s.e.mean. *P<0.05, 2-way ANOVA followed by Bonferroni post-test.

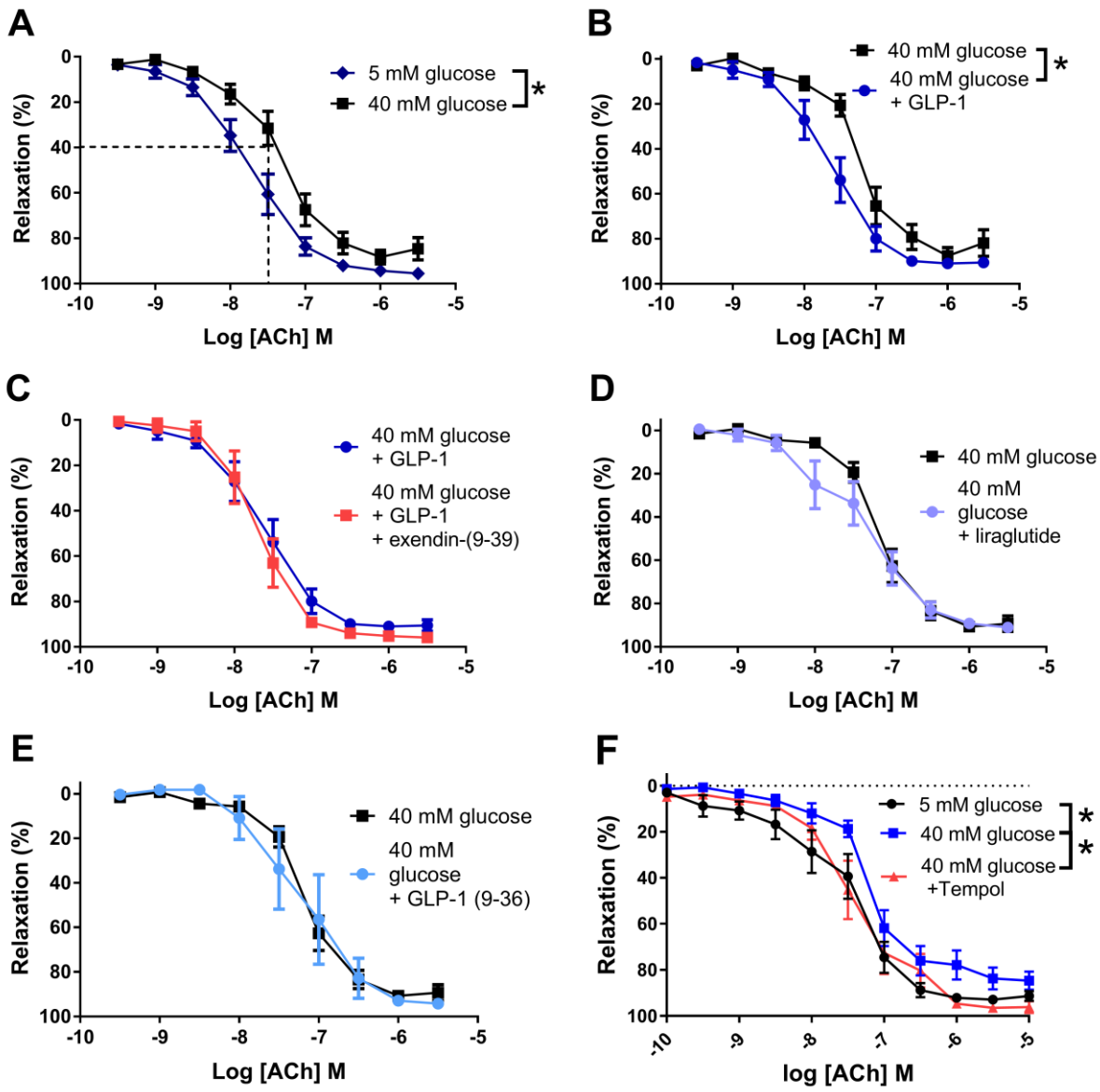

Figure 5: GLP-1 potentiates acetylcholine-induced relaxation in rat mesenteric arteries kept in high glucose. Concentration-response curves for acetylcholine (ACh) in rat meseneric arteries without branches kept (A) at 5.5 mM (n=13) and 40 mM (n=15) glucose, (B) at 40 mM glucose in the absence (n=11) and in the presence of GLP-1 (10^{-8} M, n=12), (C) at 40 mM glucose in the presence of GLP-1 (10^{-8} M) without (n=11) and with (n=4) exendin-(9-39), (D) at 40 mM glucose in the absence (n=9) and the presence of liraglutide (10^{-8} M, n=10), (E) at 40 mM glucose in the absence (n=10) and the presence of GLP-1 (9-36) (10^{-8} M, n=5). (F) at 40 mM glucose in the absence (n=10) and the presence of the superoxide dismutase mimetic, tempol (10^{-4} M, n=10), and at 5.5 mM glucose (n=10). Results are means ± s.e.mean. *P<0.05, 2-way ANOVA followed by Bonferroni post-test.

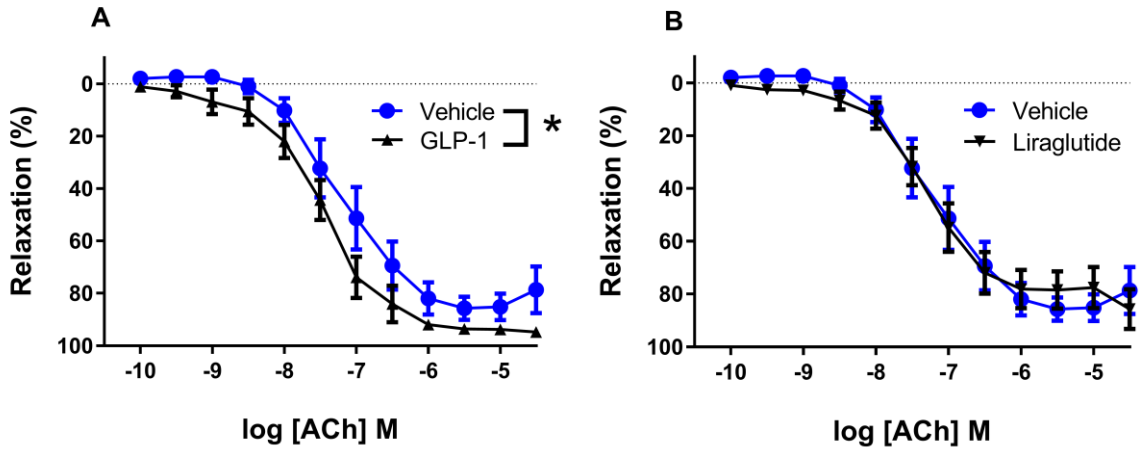

Figure 6. GLP-1 potentiates acetylcholine-induced relaxation in rat mesenteric arteries exposed to the superoxide generator, pyrogallol. Concentration-response curves for acetylcholine (ACh) in rat mesenteric arteries without branches exposed to the superoxide generator, pyrogallol ($3x10^{-5}$ M) (A) in the absence (n=10) and the presence of GLP-1 (10^{-8} M, n=10) or (B) in the absence (n=10) and the presence of liraglutide (10^{-8} M, n=10). The experiments were performed in the presence of indomethacin (10^{-6} M). Results are means ± s.e.mean. *P<0.05, 2-way ANOVA followed by Bonferroni post-test.

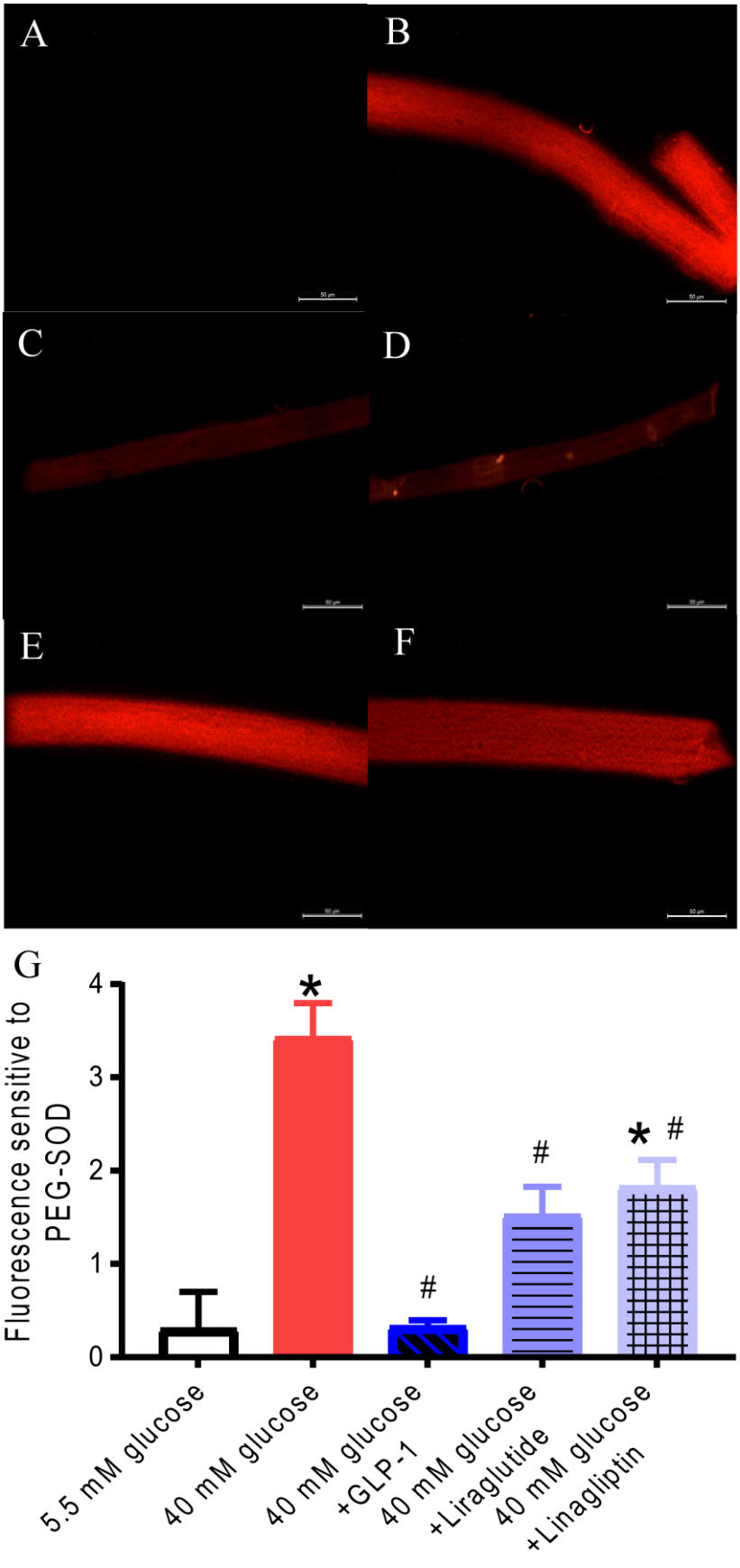

Figure 7. Dihydroethidium staining of rat mesenteric arteries is increased by high glucose and decreased by GLP-1. Representative fluorescent photomicrographs of mesenteric arteries incubated in (A) normoglycemic conditions (5.5 mM glucose), and (B) 40 mM glucose, (C) 40 mM glucose and PEG-SOD (500 U L^{-1}), (D) 40 mM glucose and GLP-1 (10^{-8} M), (E) 40 mM glucose and liraglutide (10^{-8} M), or (F) 40 mM glucose and linagliptin (10^{-6} M). White bars correspond to 50 µm. (G) Average results for dihydroethidium staining obtained in arteries from 9 animals. Columns are means (n=9) ± s.e.mean. *P<0.05 versus 5 mM glucose, # P<0.05 versus 40 mM glucose exposed vessels using one-way ANOVA followed by Tukey's multiple comparisons test.

Table 1 – Clinical characteristics of included patients

Variables	Study population (N = 23)
Age (years)	72,5 ± (8.8)
Male gender	14 (61%)
Blood glucose (mM)	7.1 ± (3.3)
Systolic Blood pressure (mmHg)	145.7 ± (16)
Diastolic blood pressure (mmHg)	77.3 ± (13.6)
Cholesterol (mM)	4.3 ± (1.1)
Smokers	12 (52%)
Previous smokers	23 (100%)
DM II	9 (39%)
Peripheral artery disease (PAD)	15 (65%)
Hypertension	13 (57%)
Lipid-lowering therapy	21 (91%)
DPP-4 inhibitor treatment	0 (0%)

Data are means ± SD or n (%).