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Decreased expression of the GLP-1 receptor after segmental artery injury in mice

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Abstract

The glucagon-like peptide-1 receptor (GLP1R) is expressed in the renal vasculature and known to be downregulated under hypertensive conditions in rats and humans. However, little is known about the regulation in other types of renal pathology involving vascular changes. This study investigates the expression of the GLP1R in renal vasculature after glomerular injury in the nephrotoxic nephritis mouse model, high cholesterol, and atherosclerosis in the *Ldlr*^{-/-} mouse on Western diet, and *ex vivo* injury in an organ culture model. The immunohistochemical signal of the GLP1R was significantly decreased in arteries from mice with nephrotoxic nephritis after 42 days compared to 7 days and saline control ($P < 0.05$). Histological evaluation of kidneys from *Ldlr*^{-/-} mice on Western diet showed a decreased GLP1R specific immunohistochemical signal ($P < 0.05$). The dilatory response to liraglutide was decreased in Western diet fed *Ldlr*^{-/-} mice compared to C57Bl/6J controls ($P < 0.05$). Organ culture significantly decreased the immunohistochemical signal of the GLP1R ($P < 0.05$) and the expression of *Glp1r* mRNA ($P < 0.005$) compared to fresh. Organ cultured vessels showed vascular smooth muscle cell remodelling as *Acta2* expression was decreased ($P < 0.005$) and *Ednrb* was increased ($P < 0.05$). In conclusion, nephrotoxic nephritis and hypercholesterolaemia led to decreased GLP1R specific immunohistochemical signal. *Ex vivo* vascular injury in the organ culture model leads to a decrease in expression of GLP1R expression and contractile VSMC specific markers and increase in expression of dedifferentiation markers suggestive of an inverse relationship between phenotypic switch of the VSMC and the expression of the GLP1R; however, the causal relationship remains elusive.

Key Words

- ▶ GLP-1 receptor
- ▶ renal artery injury
- ▶ organ culture
- ▶ nephrotoxic nephritis

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Introduction

Kidney disease has serious impact on quality of life for patients and confers severe costs on health care systems (Luyckx *et al.* 2018). Acute kidney disease is often caused by conditions such as glomerulonephritis, volume depletion, and toxic insults. Acute disease may progress to chronic kidney disease (Chawla & Kimmel 2012, Alscher

et al. 2019). Additional risk factors for development of chronic kidney disease include diabetes, metabolic disease, and hypertension (Briet & Burns 2012). The initial phase of kidney disease or injury involves changes in the renal vasculature including loss of capillaries (Choi *et al.* 2000, Horbelt *et al.* 2007). Ischaemia and resulting

activation of inflammatory pathways induce fibrosis and the transition into a chronic stage injury (Yuan *et al.* 2003, Humphreys *et al.* 2010). Vascular smooth muscle cells (VSMC), endothelial cells, and pericytes in the renal vasculature react to stimuli such as sustained increased blood pressure or persistent inflammation by vascular remodelling including dedifferentiation of VSMC (Eberhardt & Pfeilschifter 2007, Briet & Burns 2012). In recent years, clinical and preclinical studies have indicated beneficial effects of treatment with long acting GLP-1 receptor (GLP1R) agonists on renal (Moreno *et al.* 2002, Yu *et al.* 2003, Bisgaard *et al.* 2016, Mann *et al.* 2017) and cardiovascular disease (Marso *et al.* 2016ab). GLP-1 is a gut derived peptide hormone released upon a carbohydrate rich meal to the blood stream where it is rapidly broken down by dipeptidyl peptidase IV resulting in a half-life in plasma of approximately 2 min (Holst 2007). The peptide activates GLP1R in several tissues including, but not limited to, the pancreas, ventricle, and the CNS where it leads to insulin secretion, reduces gastric motility, and induces satiety, respectively (Doyle & Egan 2007, Holst 2007, Shah & Vella 2014). In the kidney, GLP1R is expressed in small and larger renal arteries where it is located on the VSMC (Pyke *et al.* 2014, Richards *et al.* 2014, Jensen *et al.* 2015) in both mouse and man (Korner *et al.* 2007, Pyke *et al.* 2014, Richards *et al.* 2014). However, the function of the GLP1R in the renal vasculature is yet to be fully understood. Studies in rodents have shown that stimulation of the renal GLP1R increases renal blood flow and perfusion, induces vasodilation of afferent arterioles, increases glomerular filtration rate, and increases diuresis and natriuresis (Moreno *et al.* 2002, Jensen *et al.* 2015, Katsurada *et al.* 2019). In humans, treatment with GLP1R agonists have been reported to induce a marginal decrease in systemic blood pressure (Marso *et al.* 2016ab). In healthy men it decreases angiotensin II secretion and aldosterone blood levels – components of the renin-angiotensin-aldosterone system, potentially indicating beneficial effects of GLP-1 on hypertension (Skov *et al.* 2013). Regulation of GLP1R expression during disease has previously been shown in the thoracic aorta of diabetic mice (Kimura *et al.* 2017) and renal vasculature of spontaneously hypertensive rats and humans (Liu *et al.* 2015, Ronn *et al.* 2017), suggesting a relationship between the pathophysiology associated with hypertension and the expression of the GLP1R. However, whether expression of the GLP1R in renal vasculature is affected by other pathological conditions has not been fully elucidated. Effects of metabolic diseases, low-grade inflammation and vascular injury on the expression and/or function of the GLP1R can be important for

regulation of kidney perfusion, blood pressure and glomerular filtration rate regulation, and it may play a role in kidney tissue integrity (Fujita *et al.* 2014, Thomas 2017). A detailed pathophysiologic understanding of the GLP-1 system in the kidneys will provide valuable insight into the mode of action of GLP1R agonists in renal injury and vascular disease.

This study investigates the change in GLP1R specific immunohistochemical signal in the renal segmental arteries (SA) under three different conditions involving vascular pathology: the nephro-toxic nephritis model (NTN), where an immune reaction to the glomerular basement membrane leads to renal vascular pathology (Ougaard *et al.* 2018b), the *Ldlr*^{-/-} mouse fed a WD diet where hypercholesterolaemia and metabolic syndrome leads to atherosclerosis (Ishibashi *et al.* 1993, Ma *et al.* 2012), and an *ex vivo* over-night organ culture model mimicking acute vascular injury (De Mey *et al.* 1989). Furthermore, real-time qPCR was employed to investigate the effect of organ culture on transcription of the *Glp1r* gene and genes related to VSMC differentiation in SA. Lastly, the change in isometric tension after stimulation with the long acting GLP1R agonist, liraglutide, was investigated in the *Ldlr*^{-/-} model and in the organ culture model using wire myography. It was hypothesized that the GLP1R expression and function is affected by renal vascular injury resulting in decreased GLP1R-specific immunosignal, down regulation of *Glp1r* mRNA and concurrent changes in vasodilatory response of the SA to liraglutide.

Materials and methods

Animal studies

The study was approved by the Danish Animal Experiments Inspectorate under the Ministry of Environment and Food and carried out by trained and licensed personnel. All animals were kept in 12 h light : 12 h darkness cycle with access to water and chow diet *ad libitum* (Altromin #1324, Brogaarden, Lyngby, Denmark) except one *Ldlr*^{-/-} group that received Western diet (*n* = 12, D12079R, Research diet, New Brunswick, NJ, USA) *ad libitum* for 21 weeks. Temperature and humidity were maintained at 22 ± 2°C and 50 ± 20%, respectively. Group sizes were chosen based on results from previous experiments.

Nephrotoxic nephritis (NTN)

NTN was induced in female CD1 mice (Charles River) by i.v. injection with sheep serum containing antibodies

towards mouse glomerulus components as previously described (Ougaard *et al.* 2018a). NTN mice were sacrificed at days 7 ($n = 5$) and 42 ($n = 5$) post serum injection. A PBS injected healthy control was euthanized at day 42 ($n = 5$). Mice were anaesthetized with isoflurane, and once fully anaesthetized a laparotomy was performed by abdominal midline incision, and the thoracic cavity was exposed by cutting along the sternum. Animals were perfused with 0.9% NaCl with heparin (10 U/mL) through the heart. Kidneys were carefully isolated and collected after perfusion and placed in 4% formalin (Hounisen, Skanderborg, Denmark) for histologic examination (see subsequently).

Ldlr^{-/-} mice on WD

The effect of hypercholesterolaemia on vascular inflammation was investigated in the *Ldlr*^{-/-} mice (B6.129S7-Ldlrtm1Her/J, Stock #02207, Jackson labs JAX) fed chow diet ($n = 11$) or WD ($n = 11$) for 21 weeks. A parallel group of healthy chow fed age matched C57BL/6J (Taconic, Lille Skensved, Denmark, $n = 10$) controlled for effect of age on vascular change. The mice were anaesthetized by s.c. injection with 0.5–0.8 mL hypnorm (fentanyl, 0.315 mg/mL and fluanisone 10 mg/mL; VetaPharma Limited, Leeds, United Kingdom) and midazolam (5 mg/mL, Accord Healthcare Limited, Middlesex, United Kingdom) mixed with sterile water 1:1:2, respectively. Once anaesthetized animals were opened as described previously and perfused by a gentle injection of approximately 10 mL ice-cold saline (Fresenius Kabi, Sèvres Cedex, France, pH 6) into the left ventricle. Immediately after perfusion the right kidney was isolated and placed in ice-cold MOPS buffered saline (5 mM KCl, 145 mM NaCl, 1 mM KH₂PO₄, 1 mM MgSO₄·7H₂O, 2.5 mM CaCl₂, and 5mM glucose, 1% albumin from Sigma, and 2 mM NaPyruvate from Gibco, adjusted to

pH 7.4 at 4°C) for dissection and myograph investigation. The left kidney was halved in the transverse plane and placed in 4% formalin (Hounisen, Skanderborg, Denmark) for histological examination.

Termination of mice for organ culture

All mice for organ culture were acclimatized for 5 days before they were picked in random order and euthanized by cervical dislocation, and kidneys were collected and placed in ice-cold MOPS buffered saline for dissection of the SA. The organ culture experiment comprises three sub-experiments. First, the acute change in *Glp1r* mRNA transcription, contractile VSMC marker α -smooth muscle cell action (*Acta2*), and VSMC dedifferentiation marker ET_B receptor (*Ednrb*) was investigated in a 6-h organ culture of SA from naïve C57BL/6J mice (Taconic, Lille Skensved, Denmark) by qPCR analyses ($n = 8$, see details subsequently). Secondly, the effect of 24-h organ culture on the response of the SA to the ET_B receptor specific agonist, sarafotoxin 6c (S6c), and the response to cumulative concentration of the long acting GLP1R agonist, liraglutide, was investigated in *Glp1r*^{-/-} mice ($n = 8$) and WT littermates ($n = 8$). *Glp1r*^{-/-}, and corresponding WT mice were custom bred by Taconic, Lille Skensved Denamrk, as described previously (Scrocchi *et al.* 1996). The background strain was C57Bl6/J.

Table 1 gives an overview of the different mice used for the three separate experimental setups in the present study.

Dissection and isometric force measurements of segmental arteries

The isolated kidneys were cleared from surrounding tissue, and the capsule was removed. SAs were dissected by cutting the kidney in half in the coronal plane so that the

Table 1 Mouse strain specifications.

Strain	Exp. setup	Age at termination	Total, n	Feed	Housing	Gender	Euthanasia
CD1	NTN	15–16 weeks	15	Chow	8/box	Female	Inh. anaesthesia and saline perfusion
<i>Ldlr</i> ^{-/-}	Extra renal vascular inflammation	46 weeks	22	Chow or WD	Single	Male	Inj. anaesthesia and saline perfusion
C57BL/6J AM	Extrarenal vascular inflammation	42 weeks	10	Chow	8/box	Male	Inj. anaesthesia and saline perfusion
Naïve C57BL/6J	Organ culture	9–13 weeks	32	Chow	8/box	Male	CD
<i>Glp1r</i> ^{-/-}	Organ culture	Appx 20 weeks	8	Chow	Single	Male	CD
<i>Glp1r</i> WT	Organ culture	Appx 20 weeks	8	Chow	Single	Male	CD

Appx, approximately; CD, cervical dislocation; Inh., inhalation; Inj, injection; NTN, nephrotoxic nephritis.

papilla and the pelvis of the kidney could be visualized. Following removal of the papillae and the segmental vein, one or two SAs were dissected and cut into segments of 1.5–2 mm length. Two 15 μ m W wires (DMT, Århus, Denmark) were carefully introduced through the lumen of the vessel, and the SA was mounted in the myograph bath (M620, DMT, Århus, Denmark) in pre gassed (5% CO₂ and 95% O₂) ice-cold physiological saline solution (PSS, 4.7 mM KCl, 119 mM NaCl, 20 mM NaHCO₃, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄·7H₂O, 2.5 mM CaCl₂, and 5 mM glucose, 0.02 mM EDTA from Sigma). After mounting the PSS was changed to pre warmed (37°C) and pre gassed (5% CO₂ and 95% O₂) PSS, and the baths were kept at a controlled temperature at 37°C and were continuously gassed with 5% O₂ and 95% CO₂.

After mounting, the SAs were normalized to 90% of the internal circumference that the vessel would have had after application of a transmural pressure of 100 mmHg, using the DMT normalization module for Lab Chart (AD Instruments, Bella Vista, Australia). Following 40 min equilibration all vessels were subjected to a wakeup protocol of three consecutive potassium responses using 63 mM KCl PSS (63 mM KCl, 55.5 mM NaCl, 20 mM NaHCO₃, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄·7H₂O, 2.5 CaCl₂ all from Merck, and 5 mM glucose, 0.02 mM EDTA from Sigma). A threshold of 0.5 mN/mm constriction in the last KCl response was the inclusion criteria through all myograph experiments. The following agents were used to produce concentration response curves: phenylephrine (PE, 10⁻¹⁰–10⁻⁴M, Sigma, stock solution 0.1 M in Milli-Q), sarafotoxin6c (S6c, 10⁻⁷M, Tocris, Abingdon, UK, stock solution 0.1 M in 0.1% human serum albumin). For pre-constrictions and time control curves a fixed concentration of PE 10⁻⁶M was used (Sigma, stock solution 0.1 M in Milli-Q). Dilatory substances were added to the pre-constricted vessels to assess endothelium dependent vasodilation by acetylcholine (Ach, 10⁻¹⁰–10⁻⁴ M, Sigma, stock solution 0.1 M in Milli-Q) and endothelium independent NO mediated vasodilation by sodium nitroprusside (SNP, 10⁻¹⁰–10⁻⁵ M, Sigma, stock solution 0.1 M in Milli-Q). All stocks were kept at -20°C. For studies of GLP1R mediated dilation by liraglutide in 0.007% polysorbate 20, 50 mM PO₄, 70 mM NaCl and Milli-Q water (stock concentration 1 μ M and kept at 4°C, Novo Nordisk A/S), human serum albumin was added to buffer to reach concentration of 0.1%, and vessel was allowed to stabilize before liraglutide was added in accumulating doses with at exactly 7 min intervals (10⁻¹¹–10⁻⁵M). Prior to the liraglutide concentration response a 42 min time control was run on each artery to control for variation in the PE pre-constriction during the

very long liraglutide response. After the time control, the artery was washed thrice and allowed to rest for 30 min before pre-constricting with 10⁻¹⁰ M PE for the liraglutide concentration response. The time control curve change in tension from initial precontraction corresponding to each time point in the liraglutide concentration response was subtracted the liraglutide response.

Organ culture

For organ culture experiments, a second segment was dissected from the animal from which the fresh vessel was isolated. The segment was mounted onto one 15 μ m W wire and transferred to a 24 well plate with 1 mL serum free medium DMEM (DMEM1X+GlutaMAX™, 1 g/L Glucose, +pyruvate, Gibco life technologies) mixed with Pen Strep (penicillin 100 IU/mL, streptomycin 100 μ g/mL, respectively, Gibco Life technologies). Plates were placed in incubator of 37°C with humidified 5% CO₂ in air for 24 h (+/1 h). OC vessels were mounted the following day on a second wire in the myograph baths in preheated (37°C) and pre gassed (5% CO₂, 95% O₂ for 20 min) PSS and subjected to a protocol identical to the fresh vessel experiment to ensure pairing of the two vessels from the same animal.

qPCR

For mRNA expression of segmental arteries in organ culture, RNA was isolated from collected SAs by phase separation with trizol (15596026, Ambion) and 1-bromo-3chloropropane (1-BCP, Sigma). RNA was extracted from the aqueous phase using the RNeasy micro kit (Qiagen). RNA concentration was analysed by NanoDrop 8000 system (Thermo Fisher Scientific). cDNA was prepared by iScript cDNA synthesis kit (BioRAD). qPCR was run on 2.5 $\times$ diluted cDNA using Taqman Fast Advanced Master mix (PN 4444557, Life Technologies) and Taqman Gene Expression Assays for the three genes coding for the GLP1R, *Glp1r* (Mm00445292_m1). The effect of OC on VSMC dedifferentiation and differentiation was investigated by regulation of the ET_B receptor coded for by *Ednrb* (Mm00432989_m1), which was used as a marker of VSMC dedifferentiation and the contractile SMC marker α SMA coded for by *Acta2* (Mm00725412_s1) as marker of differentiated VSMC. Actin beta (*Actb*, Mm02619580_g1) was used as reference gene (All from Life Technologies).

Immunohistochemistry

Fixed kidneys were halved in the transverse plane, dehydrated, and embedded in paraffin. For preparation

of dissected SAs the arteries were first embedded in 3% Bacto™ Agar (BD Diagnostics, Franklin Lakes, New Jersey, USA), dehydrated, and embedded in paraffin for microtome sectioning (4.5 µm). Sections were stained with haematoxylin eosin (HE) according to standard procedures. Immunohistochemical stains were performed using the Ventata Discovery XT automation system (Ventana Inc., Roche), and the Rb-GLP1R antibody (2.7 µg/mL, ab218532 Abcam) labelled using the DISCOVERY purple chromogen kit (ref: 760-229, Roche). Please refer to Supplementary Fig. 4 (see section on [supplementary materials](#) given at the end of this article) for specificity to the mouse GLP1R. An identical protocol without primary antibody was run in parallel as negative control on an adjacent level to the level stained for GLP1R. The ratio of immunohistochemical stain area per arterial cross section area was quantified using the HALO software as previously described (Koelzer *et al.* 2018) and shown in per cent of vessel wall area (algorithm: Area of Quantification version 2.31, Indica Labs, Albuquerque, USA). To standardize vessel type and size and ensure comparability to the SA isolated after myograph, a subset of arteries from whole kidney sections (3–10 per kidney dependent on availability on specific section) were selected. As diameter could be affected by disease and technical circumstances after myograph mounting, the diameter of the vessel selection based on diameter might introduce bias. Consequently, the anatomical selection criterion of the relevant arteries was that the muscularized artery was located adjacent to a vein and not surrounded by perivascular adipocytes corresponding to the SA or the first branch hereof (*A. interlobare*). The positive immunohistochemical staining is given as a ratio of the cross section of that specific vessel. The results of one mouse represents the mean ratio of all analysed arteries in one cross-sectional level. All image analyses were carried out in a blinded fashion.

Modelling and statistics

All data are presented as mean ± S.E.M. Data were analysed using GraphPad Prism version 8 (Graph Pad). Outliers were identified using the ROUT test and removed (one outlier was removed based on this test) (Motulsky & Brown 2006). For statistical analyses, values of $P < 0.05$ were considered significant. Normal distribution of data was tested with Shapiro Wilk's test, in case data were not normally distributed a non-parametric test was used as follows: for comparisons between two groups Student's *t*-test or Wilcoxon paired rank test were applied for parametric and non-parametric data, respectively.

For comparisons between more than two groups an Ordinary One-Way ANOVA followed by Tukey's multiple comparison test or Kruskal–Wallis test followed by Dunn's multiple comparisons test was applied for parametric and non-parametric distributions, respectively. Organ culture data were considered paired as fresh and organ culture vessel originated from the same animal.

Results

GLP1R immunohistochemical detection in NTN model of renal injury

Representative images of immunohistochemistry (IHC) of GLP1R in NTS dosed CD1 mice are displayed in Fig. 1A, B, C, D, E and F. NTS led to changes in renal tissue architecture characterized by lack of glomerulus tubule structure, interstitial tissue oedema and infiltration with inflammatory cells, the vascular wall thickness was increased at 42 days post-NTS injection (Fig. 1A, B, C, D, E, F and Supplementary Fig. 1). GLP1R positive signal was observed in the VSMC, whereas the endothelium and remaining structures in the kidney that is, tubules, interstitium and calyces were negative of IHC signal. A time dependent decrease in GLP1R specific signal in the vessel wall was observed with most pronounced reduction of the signal 42 days after injection of NTS (Fig. 1B, D and F). No positive stain was detected in the vasculature of the negative control (Fig. 1G). Semiquantitative analyses of the IHC signal in the arteries showed a time dependent decrease of the GLP1R specific signal in the renal arteries (Fig. 1H) with a significant difference between controls and NTS at 42 days ($P = 0.0054$) and between 7 and 42 days post NTS injection ($P = 0.0460$).

GLP1R detection and function in *Ldlr*^{-/-} mouse model

Renal tissue from C57Bl/6J AM and *Ldlr*^{-/-} chow fed animals showed intact architecture and positive detection of GLP1R signal (Fig. 2A and B). Kidneys of WD fed mice showed marked perivascular accumulation of inflammatory cells in 5 out of the 11 *Ldlr*^{-/-} mice on WD (Fig. 2C and Supplementary Fig. 2). Negative controls, without primary antibody showed no positive stain (Fig. 2D). Arteries of kidneys from *Ldlr*^{-/-} mice on WD showed a significant decrease in GLP1R specific staining intensity compared to age matched C57Bl/6J (Fig. 2E, $P = 0.0404$) and a tendency to a decreased staining compared to chow fed *Ldlr*^{-/-} animals (Fig. 2E, $P = 0.136$).

Figure 1

Results of immunohistochemical detection of GLP1R in NTN mice. (A, B, C, D, E and F) Representative images of GLP1R IHC from healthy PBS injected animals (top row), 7 days after injection of NTS (second row), and 42 days after NTS injection (third row). In each panel: first column 10 $\times$ magnification of kidney tissue architecture, scale bars 500 μ m. Second column 40 $\times$ magnification of black square, scale bars 100 μ m. Arrowhead, loss of regular glomerulus structure, long arrow: interstitial oedema, short arrow: infiltration with inflammatory cells. (G) 40 $\times$ magnification of negative control. *a*, artery; *v*, vein; *Fe*, iron sediments in tubules; *i*, perivascular inflammatory cells. (H) Results of image analysis of area of quantification of selected arteries from kidney preparation from PBS ($\bullet$, $n = 5$) or NTS injected mice at days 7 ($\blacksquare$, $n = 5$) and 42 ($\blacktriangle$, $n = 5$) after injection. Individual values represent mean of 3–9 arteries per mouse. Data shown as individual values, mean and S.E.M. Ordinary One-Way ANOVA, Tukey's *post hoc* analysis. * $P < 0.05$, ** $P < 0.005$.

There were no differences in vasomotor responses to 63 mM KCl, PE, Ach, and SNP or passive diameter of the SA between the three groups (Table 2 and Supplementary Fig. 3). WD fed animals had significantly higher bodyweight, higher blood cholesterol, and blood triglycerides. These results are published elsewhere (Bjørnholm *et al.* 2020). Liraglutide exposure (10^{-6} M) induced a vasodilatory response on PE pre-constricted vessels. The dilatory response to 10^{-6} M liraglutide was significantly attenuated in SA from *Ldlr* $^{-/-}$ mice on WD compared to C57BL/6J aged-matched controls (Fig. 2F, $P = 0.0419$).

Effect of organ culture on GLP1R gene expression, immunohistochemical staining and function

Fresh vessels were characterized by a strong stain of GLP1R in the vessel wall (purple, Fig. 3C) and organ cultured exhibited a lower GLP1R immunoreactivity (Fig. 3D). Quantification of the stain area ratio (of vessel wall cross section) resulted in a significant decrease of GLP1R specific immunoreactivity after 24 h organ culture (Fig. 3E, $P = 0.0213$). Organ culture of 6 h led to significant downregulation of *Glp1r* gene expression compared to fresh vessels (Fig. 3F, $P = 0.0006$). Liraglutide induced a concentration dependent relaxation of pre-constricted

SA from WT mice whereas the relaxation was absent in *Glp1r* $^{-/-}$ mice confirming that the relaxation was mediated by a GLP1R specific mechanism both in fresh and organ cultured vessels (Fig. 4A and B). Trace graphs of the liraglutide response (Fig. 4A and B, respectively) showed a highly variable and oscillating nature of the liraglutide response in organ culture SA compared to fresh, where the response was consistent and uniform. Thus, whereas the vasodilatory response to liraglutide appears to be qualitatively changed after organ culture attaining a much more oscillatory nature than in fresh SA, the oscillatory nature of the liraglutide responses after organ culture prevented quantitative comparisons between the magnitude of the response in fresh vs organ cultured SA.

Effect of organ culture on VSMCs phenotypic switch

Organ culture of segmental arteries from naïve C57BL/6J mice induced significant increase in expression of the ET_B receptor (*Ednrb*, Fig. 5A, $P = 0.0313$) and a significantly lower expression of the contractile smooth muscle cell marker *Acta2* (Fig. 5B, $P = 0.0018$), suggesting a shift to a dedifferentiated VSMC phenotype in the organ cultured vessels. Fresh SA from *Glp1r* $^{-/-}$ showed a significantly larger contractile response to the selective ET_B receptor agonist sarafotoxin 6c (S6c, 10^{-7} M) compared to fresh SAs

Figure 2

Regulation of the GLP1R in the *Ldlr*^{-/-} mouse. (A, B and C) Representative images of C57BL/6J AM controls (top row), *Ldlr*^{-/-} on chow (second row), and *Ldlr*^{-/-} on WD (third row) and negative controls (last row). Arrows point to accumulated inflammatory cells, 40× magnification of GLP1R expression in artery, scale bar 100 µm. (D) Negative control, no primary antibody. (E) results from IHC image analyses of GLP1R stain area of selected arteries from C57BL/6J AM (●, *n* = 9), *Ldlr*^{-/-} Chow (■, *n* = 10), and *Ldlr*^{-/-} WD (▲, *n* = 11). Data shown as individual values, mean and s.e.m. Kruskal Wallis test and Dunn's multiple comparison test. (F) Response of isolated SA in wire myograph to 10⁻⁶M liraglutide from. Data shown as individual values, mean and s.e.m. Ordinary One-way ANOVA, Tukey's multiple comparison test. **P* < 0.05 PE, phenylephrine; IHC, immunohistochemistry; OC, organ culture; SA, segmental artery; AM, age matched; WD, Western diet.

from WT mice (Fig. 5C). Twenty-four hours organ culture significantly increased the contractile response to S6c in both WT and *Glp1r*^{-/-} (Fig. 5C, *P* < 0.0001). Myograph investigation of fresh and organ culture SA showed no significant change in 63 mM KCl induced constriction of SA in the myograph after organ culture (Fig. 5D).

Organ culture did not affect the contractile response to PE or the passive diameter. There was a significant increase in ET-1 induced constriction (*P* = 0.0403) (Table 3).

Discussion

The present study shows that the GLP1R specific immunohistochemical signal is decreased in renal arteries of two *in vivo* mouse models of chronic vascular injury: (i) the NTN model, a renal specific injury model where mice develop kidney damage by an immune-mediated reaction to injected NTS (Ougaard *et al.* 2018b), and (ii) the Western diet fed *Ldlr*^{-/-} model, a model of hypercholesterolaemia and atherosclerosis also known to develop prediabetic metabolic disturbances (Ishibashi *et al.* 1993, Foote *et al.* 2016). Perivascular accumulation of inflammatory cells in the kidney was present in both models suggesting that inflammation plays a role in the vascular changes reported. Inflammation and renal vascular remodelling are well documented in rodent models of chronic kidney disease (Yuan *et al.* 2003, Horbelt *et al.* 2007, Basile *et al.* 2011, Briet & Burns 2012, Silva *et al.* 2019), whereas the *Ldlr*^{-/-} mouse is not commonly known for its renal pathology. *Ldlr*^{-/-} mice on WD are known to exhibit chronic low-grade inflammation, which is thought to contribute to the atherosclerotic changes seen in the model (Inoguchi *et al.* 1992, Foote *et al.* 2016). Atherosclerosis and metabolic conditions such as elevated cholesterol, obesity, and type two diabetes are known to increase the risk of chronic kidney disease (Carmines 2010). This may be a two-way interaction between vascular disease and kidney disease as moderately uremic (by partial nephrectomy) WD fed *Ldlr*^{-/-} mice showed increased atherosclerotic burden compared to sham operated controls (Bisgaard *et al.* 2016). Bisgaard *et al.* show that liraglutide treatment in moderately uremic mice decrease renal damage and atherosclerosis compared to WT controls. In patients with renal disease increases in atherosclerotic burden has also been reported (Preston *et al.* 2005, Valdivielso *et al.* 2019) as has attenuation of renal disease progression by treatment with liraglutide in patients with type 2 diabetes (Mann *et al.* 2017). This supports the results presented here that GLP1R activation is important for vascular responses to renal injury.

The GLP1R has previously been reported to mediate increased renal flow and perfusion by vasodilatory responses of VSMC associated GLP1R activation (Moreno *et al.* 2002, Jensen *et al.* 2015), consistent with the vasodilator response to GLP1R stimulation with

Table 2 Vasomotor function of SA from *Ldlr*^{-/-} mice.

Response		C57Bl/6J		<i>Ldlr</i> ^{-/-} chow		<i>Ldlr</i> ^{-/-} WD		P-value (OOW ANOVA)
		n	Mean (S.E.M.)	n	Mean (S.E.M.)	n	Mean (S.E.M.)	
Diameter	μM	10	239 (10.4)	11	249 (12.3)	11	267 (13.0)	0.275
KPSS (63 mM) (mN/mm)	E _{max}	10	3.42 (0.321)	11	3.49 (0.459)	11	3.28 (0.329)	0.919
PE CRC (mN/mm)	E _{max}	10	3.74 (0.146)	11	3.85 (0.181)	11	3.86 (0.167)	0.972
	LogEC ₅₀		-6.88 (0.0816)		-6.84 (0.0958)		-6.70 (0.0883)	
Ach CRC (% dilation)	E _{max}	10	65.5 (2.32)	11	63.8 (4.70)	11	73.36 (3.30)	0.792
	LogEC ₅₀		-7.18 (0.0675)		-6.83 (0.119)		-6.85 (0.0720)	
SNP CRC (% dilation)	E _{max}	10	80.7 (1.80)	11	78.5 (2.44)	11	77.3 (2.49)	0.945
	LogEC ₅₀	10	-7.17 (0.0500)	11	-7.08 (0.0746)	11	-7.00 (0.0759)	

Ach, acetylcholine; CRC, concentration response curve; OOW ANOVA, ordinary one-way analyses of variance; PE, phenylephrine; SNP, sodium nitroprusside.

liraglutide in SA from healthy animals reported in the present study. However, Ronn *et al.* found an increase in mean arterial pressure, renal blood flow, and natriuresis after infusion of GLP-1 despite the absence of *Glp1r* in the renal arteries of spontaneously hypertensive rats (Ronn *et al.* 2017) suggesting that extrarenal GLP-1-induced effects, may at least in part be responsible for the blood pressure effects of *Glp1r* activation in rodents and not directly from GLP-1 mediated vasodilation of

renal arteries. Consequently, it may be speculated that activation of the *Glp1r* in the renal vasculature promotes other physiological processes important in the healthy kidney, but independent of vasodilation and blood pressure regulation. Previously, it has been shown that GLP-1 treatment has an anti-inflammatory effect, which could be beneficial in clinical and preclinical kidney disease (Hogan *et al.* 2014, Ougaard *et al.* 2020). It can be argued that a decrease in renal GLP1R lowers the

Figure 3

Effect of organ culture on expression of *Glp1r*. (A) Representative HE stains from fresh SA, (B) representative HE stains of organ culture SA. (C and D) *Glp1r* IHC stain (purple) in organ culture SA corresponding to HE examples. (E) Results of image analyses of *Glp1r* positive stain area from fresh (●) and organ culture 24 h (■) SA. Paired Student's *t*-test. **P* < 0.05, ***P* < 0.005. (F) qPCR results of *Glp1r* expression from fresh (●) and organ culture 6 h (■) SA. Individual values, mean and S.E.M. Paired Student's *t*-test. (G) Negative control from fresh SA. All images are captured at 40× magnification. Scale bars 100 μm. IHC, immunohistochemistry.

Figure 4

Liraglutide functional response in fresh and organ culture SA from *Glp1r*^{-/-} and WT. (A) Trace graph averaging every 4 s of the recorded liraglutide response respective to concentration exposure from fresh SA from WT (■) and *Glp1r*^{-/-} (▼) mice, data are shown as mean and S.E.M., liraglutide concentration given above. (B) Trace graph of organ cultured SA from WT (■) and *Glp1r*^{-/-} (▼) mice, data are shown as mean and S.E.M., liraglutide concentration given above. PE, phenylephrine; Conc., concentration.

Figure 5

Effect of organ culture on markers of VSMC dedifferentiation and ET_B receptor response to S6c. (A) *Ednrb* (ET_B receptor) expression from fresh (●) and organ cultured (■) SAs from naïve C57BL/6J mice. Individual values, mean and s.e.m. Wilcoxon paired rank test. (B) *Acta2* expression in the same SAs as A. Individual values, mean and s.e.m. Student's *t*-test. (C) Response to ET_B receptor agonist, sarafotoxin 6c (S6c), in SA from *Glp1r*^{-/-} and WT littermates (same experiment as Fig. 4). Given as isometric tension relative to last 63 mM KCl response in myograph protocol. Data are shown as individual values, mean, and s.e.m. Two-way ANOVA, Sidak's multiple comparison. (D) Contractile response to 63 mM KCl in same vessels as C. Data given as individual values, mean and s.e.m. No difference detected under the two-way ANOVA. SA, segmental artery; S6c, sarafotoxin 6c; WT, wild type; OC, organ culture. **P* < 0.05, ***P* < 0.005, ****P* < 0.0005.

protective effect of native GLP-1 on vascular health. The effect is restored by supra-physiological concentrations of GLP1R agonists (such as liraglutide), which activates the remaining GLP1Rs and re-establish the vascular protective effects of GLP1R activation contributing to an overall attenuation of renal disease.

To test if direct vascular injury changes the expression level and vasodilatory function of the GLP1R in the SA, organ culture was used as a model of injury in a system devoid of extravascular injury response mechanisms. Organ culture of the SA from naïve mice induced a rapid decrease (6 h) in *Glp1r* mRNA expression, a decrease in the expression of the contractile VSMC specific marker *Acta2* and an increase in expression of *Ednrb* coding for the ET_B receptor. In rats, ET_B receptors are expressed in kidney VSMCs of afferent arterioles mediating contraction and by endothelial cells of the efferent arteriole mediating vasodilatation, thus playing an important role in maintenance of vascular tone (Inscho *et al.* 2005). The contractile ET_B receptor is known to be upregulated in the dedifferentiated VSMC phenotype *in vivo* after acute vessel injury and in organ culture (Adner *et al.* 1998, Krawczyk *et al.* 2018, Skovsted *et al.* 2019). The time point of 6 h was based on the rapid reaction of VSMC in organ culture leading to changes in transcription of the ET_B receptor previously shown in coronary arteries from rats (Skovsted *et al.* 2012). Twenty-four hours organ culture resulted in a decrease in GLP1R specific immunostaining signal in cultured vessels compared to fresh. Change in the VMSC phenotype was confirmed after 24 h of culture by an increase in the functional response to the specific ET_B receptor agonist S6c in cultured vessels compared to fresh. Twenty hours were chosen for investigation of protein levels by immunohistochemistry to allow for the rapid transcriptional change to manifest in protein level expression and in functional responses.

Table 3 Vasomotor function in fresh and organ cultured vessels from naïve mice.

Response		Fresh		Organ culture		P-value (t-test, fresh vs organ culture)
		n	Mean (s.e.m.)	n	Mean (s.e.m.)	
Diameter (μm)		19	210 (31.3)	23	227 (30.5)	0.121
KCl (63 mM) (mN/mm)	E _{max}	19	1.91 (0.252)	23	1.76 (0.209)	0.696
PE (10 ⁻⁵ M)	E _{max}	10	2.27 (0.329)	13	1.99 (0.235)	0.441
ET-1 (10 ⁻⁷ M) (mN/mm)	E _{max}	5	1.41 (0.254)	7	2.11 (0.317)	0.0403*

Significant *P*-values are emphasized in bold.

**P* < 0.05.

Ach, acetylcholine; CRC, concentration response curve; PE, phenylephrine; SNP, sodium nitroprusside.

Data from all three models of SA artery injury suggest that GLP1R expression is connected to VSMC phenotype and vascular inflammation. Chronic vascular disease such as atherosclerosis has been shown to activate the protein-kinase C beta (PKC β) pathway in response to signals including, but not limited to, upregulation of intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) in the activated endothelium as well as inflammatory cytokines such as TNF α leading to downstream release of growth factors ultimately resulting in VSMC activation and dedifferentiation (Yan *et al.* 2006, Kong *et al.* 2013). Interestingly, the same pathway has been suggested to downregulate the GLP1R in spontaneously hypertensive rats (Liu *et al.* 2015, Ronn *et al.* 2017). The VSMC of resistance and conductance arteries from spontaneously hypertensive rats are known to show signs of remodelling and dedifferentiation (Owens *et al.* 1988, Berk *et al.* 1989), which may contribute to arterial stiffening (Hays *et al.* 2018). Moreover, VSMC from spontaneously hypertensive rats have been have increased expression and activity to protein-Kinase C activation (Silver *et al.* 1988, Peng *et al.* 2007), a pathway that is common for inflammatory vascular conditions. Hence, further investigations are warranted to confirm the role of inflammatory pathways in injury-induced GLP1R regulation in the SA.

Alternatively, the connecting pathway between GLP1R expression and VSMC phenotypic switch may involve the transcription factor TCF7L2. In the healthy vessel TCF7L2 activated by Wnt/LRP6 signalling on the VSMCs and confers the maintenance of the VSMC in a contractile state (Srivastava *et al.* 2015, 2019). In the injured vessel Wnt/LRP6 signalling is impaired which leads to activation of transcription factor SP1 resulting in VSMC phenotypic switching (Srivastava *et al.* 2015). Interestingly, Kimura *et al.* showed that TCF7L2 is also involved in GLP1R downregulation in VSCM in diabetic mice (Kimura *et al.* 2017). Considering this in light of the present data it could be speculated that the decrease in GLP1R expression in SA during vascular injury is causally linked to VSMC dedifferentiation mediated by a common transcriptional pathway involving TCF7L2. The fact that fresh SA from *Glp1r*^{-/-} mice show an increased response to the ET_B receptor agonist S6c supports that GLP1R signalling is indeed involved in the regulation of VSMC phenotype, albeit more investigations are necessary to establish the precise causal relationship.

In agreement with findings with native GLP-1 (Jensen *et al.* 2020), this study shows that liraglutide results in a

receptor-mediated dilatory response in the SA of *Ldlr*^{-/-} mice and naïve C57BL/6J mice. Chronic vascular injury decreased the dilatory response to liraglutide in the wire myograph investigation of the SA from *Ldlr*^{-/-} mouse on WD despite that there were no changes in the SA response to the other vasodilators Ach and SNP.

Based on the present results from NTN and *Ldlr*^{-/-} mice it cannot be concluded if the decreased GLP1R specific IHC signal is due to a downregulation of receptor expression or increased receptor activity and subsequent internalization and degradation. The GLP1R is a G-protein coupled receptor located on the membrane, which is expected to be internalized following activation, and subsequently degraded following beta-arrestin activation (Jones *et al.* 2018). As beta-arrestin is activated by cAMP levels (Parruti *et al.* 1993), and these are known to be decreased during vascular damage for example, by increase in phosphodiesterase activity (Priksz *et al.* 2018), it may be speculated that a decrease in beta-arrestin could mediate an impaired GLP1R de-sensitization resulting in maintained function during acute downregulation of the GLP1R.

The present work is a collection of data from three models representing three different mouse strains (CD1, *Ldlr*^{-/-}, C57BL/6J, and *Glp1r*^{-/-}) and also both female (NTN) and male (*Ldlr*^{-/-}, C57BL/6J, and *Glp1r*^{-/-}) mice under different housing conditions. Furthermore, three different methods of disease induction are included challenging the direct translation between magnitude of response in the different models. Thus, the main conclusion from the findings in this study is that the three different disease paradigms all lead to a significantly lower GLP1R signal suggestive of a qualitative change in GLP1R expression across a range of different renal vascular injury models.

Conclusions

The present report shows that mouse models of nephrotoxic nephritis and atherosclerosis lead to a decrease in GLP1R specific immunohistochemical signal. In the atherosclerotic *Ldlr*^{-/-} mouse on WD also showed a decrease in dilatory response to liraglutide. The organ culture model mimicking acute vascular injury *ex vivo* led to a decrease in expression of *Glp1r* and contractile VSMC specific markers and increase in expression of dedifferentiation markers. The response to the ETB receptor specific agonist S6c was greater in *Glp1r*^{-/-} compared to WT littermate controls suggestive of an

relationship between phenotypic switch of the VSMC and the expression of the GLP1R however, the causal relationship remains elusive.

Supplementary materials

This is linked to the online version of the paper at <https://doi.org/10.1530/JOE-20-0608>.

Declaration of interest

G K P, M E O, G R, and C P are employees of Novo Nordisk A/S that produces liraglutide. The remaining authors are employed by University of Copenhagen and have collaborated with Novo Nordisk on the present project.

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