A long-acting glucagon-like peptide-1 analogue attenuates induction of plasminogen activator inhibitor type-1 and vascular adhesion molecules

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Abstract

Glucagon-like peptide-1 (GLP-1) administration attenuates endothelial cell dysfunction in diabetic patients and inhibits tumour necrosis factor α (TNF)-mediated plasminogen activator inhibitor type-1 (PAI-1) induction in human vascular endothelial cells. The short half-life of GLP-1 mediated via degradation by the enzyme dipeptidyl peptidase 4 mandates the clinical use of long-acting GLP-1 analogues. The effects of a long-acting GLP-1 analogue on PAI-1 and vascular adhesion molecule expression in vascular endothelial cells are unknown. In this report, we demonstrate for the first time that the treatment with liraglutide, a long-acting GLP-1 analogue, inhibited TNF or hyperglycaemia-mediated induction of PAI-1, intercellular adhesion molecule-1 and

vascular cell adhesion molecule-1 mRNA and protein expression in a human vascular endothelial cell line. In addition, treatment attenuated TNF- or hyperglycaemia-mediated induction of the orphan nuclear receptor *Nur77* mRNA expression. Taken together, these observations indicate that liraglutide inhibits TNF- or glucose-mediated induction of PAI-1 and vascular adhesion molecule expression, and this effect may involve the modulation of NUR77. These effects suggest that liraglutide may potentially improve the endothelial cell dysfunction associated with premature atherosclerosis identified in type 2 diabetic patients.

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Introduction

Type 2 diabetes is characterized by accelerated atherosclerosis (Hobb 2006). Elevated tumour necrosis factor α (TNF) levels and hyperglycaemia are implicated in diabetes-associated endothelial cell dysfunction and may be causal in premature atherosclerosis (Morigi *et al.* 1998, Liu *et al.* 2005, 2008, Norata *et al.* 2006, Kumar *et al.* 2007, Iwasaki *et al.* 2008).

TNF and hyperglycaemia have been shown to induce plasminogen activator inhibitor-1 (PAI-1) and vascular cell adhesion molecule (ICAM-1 and VCAM-1) expression in human vascular endothelial cells (Morigi *et al.* 1998, Liu *et al.* 2005, 2008, Norata *et al.* 2006, Kumar *et al.* 2007, Iwasaki *et al.* 2008). PAI-1 and vascular adhesion molecules, including VCAM-1 and ICAM-1, are elevated in diabetes and are thought to participate in the pathogenesis of atherosclerosis (Sobel *et al.* 1998, Blankenberg *et al.* 2003). Interestingly, mice with a targeted deletion of the PAI-1 gene are protected from the development of biochemical abnormalities associated

with endothelial cell dysfunction and development of atherosclerosis (Eitzman *et al.* 2000, Mao *et al.* 2004).

Liraglutide, an acylated glucagon-like peptide-1 (GLP-1) analogue, has a half-life of 10–14 h allowing once-daily s.c. injection (Elbrønd *et al.* 2002). Clinical trials of liraglutide in patients with type 2 diabetes demonstrate improved levels of both fasting and postprandial glucose, improvement in β -cell function, ability to delay gastric emptying and a reduction in plasma glucagon concentration and weight loss (Elbrønd *et al.* 2002, Juhl *et al.* 2002, Degn *et al.* 2004, Vilsboll *et al.* 2007).

In vivo studies demonstrate that native GLP-1 regulates vascular tone and endothelial function (Golpon *et al.* 2001, Yu *et al.* 2003), and a clinical study has reported reduced endothelial dysfunction in type 2 diabetic patients with established coronary artery disease (Nyström *et al.* 2004). *In vitro* native GLP-1 attenuates TNF α -induced PAI-1 expression in human vascular endothelial cells, an effect that may utilize the orphan nuclear receptor NUR77 (Liu *et al.* 2008) and provide further evidence for GLP-1-mediated reduction in endothelial cell dysfunction.

The *in vitro* effects of liraglutide on PAI-1 and vascular adhesion molecule expression, under conditions associated with vascular endothelial cell dysfunction, have not yet been explored. Our study aims to characterize the effects of liraglutide on the regulation of PAI-1 and VCAM-1 expression under conditions of TNF stimulation or hyperglycaemia in human vascular endothelial cells and determine the aspects of the molecular mechanisms responsible for this effect.

Materials and methods

Materials and cell culture

Liraglutide was kindly provided by Novo Nordisk, Denmark. The spontaneously transformed human umbilical vein endothelial cell line, C11 STH (Cockerill *et al.* 1994) was cultured under sterile conditions. Experiments were performed at between three and six cell passages and 80% cellular confluence at 37 °C using gelatine-coated Nunclon cell culture dishes in Media-199 (Sigma) supplemented with penicillin/streptomycin, 20% FCS, 20 µg/ml endothelial cell growth factor (Sigma) and 20 µg/ml heparin. Insulin concentration in FCS was 17.0 pM. Glucose concentration in media was 6.0 mM.

Determination of PAI-1, ICAM-1 and VCAM-1 proteins

C11 STH cells were incubated under serum-free conditions with 10 ng/ml TNF (Promega) pretreatment for 2, 5 or 16 h or with 10 mM glucose for 48 h. Liraglutide (1 nM–1 µM) was added for one hour subsequent to the incubation period with TNF α or concurrently with glucose stimulation. The conditioned medium (CM) was subsequently collected and centrifuged at 10 000 revolutions per minute (r.p.m) for 1 min; the PAI-1 protein concentration in the CM was assayed using a PAI-1 ELISA (Trinity Biotech, Bray, Ireland). The ICAM-1 and VCAM-1 proteins were measured using ELISA kits (R&D Systems, Minneapolis, MN, USA). All experiments were performed with triplicate incubates and repeated thrice, independently.

Semi-quantitative reverse transcription-PCR (RT-PCR)

Total RNA was extracted by the method of Chomczynski & Sacchi (1987). RNA was heated at 65 °C for 10 min immediately prior to first-strand cDNA being generated using reverse transcriptase (Invitrogen) with oligo (dT)₁₅ primers, in the presence of 1 mM of each dNTP and DTT. For the RT-PCR step, 20 µl reaction volume was used. The primers

used for PAI-1 detection were: forward 5'-CAGACCAA-GAGCCTCTCCAC-3'; reverse 5'-ATCACTTGGCCCAT-GAAAAG-3'; VCAM-1: forward 5'-GATACAACCGTCTTGGTCAGCCC-3', reverse 5'-CGCATCCTTCAACTGGGCCTT-3'; ICAM-1: forward, 5'-CAGTGACCATCTACAGCTTTCCGG-3', reverse: 5'-GCTGCTACCACAGTGATGATGACAA-3'. The human glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene was used as an internal control. The forward primer for GAPDH was 5'-CCTGCACCACCAACTGCTTAGC-3' and the reverse primer was 5'-CCAGTGAGCTTCCCGTCTAGC-3'. All PCR products were sequenced to confirm the identities and repeated thrice.

Scanning densitometry analyses were performed on RT-PCR products using the GeneSnap imaging software (Synoptics, Cambridge, UK). Band intensity values for ICAM-1, PAI-1 and VCAM-1 mRNA were normalized to those of GAPDH mRNA and the resultant ICAM-1, PAI-1 and VCAM-1: GAPDH ratios were plotted to illustrate variations in the gene expression.

Real-time PCR

cDNA was generated as previously described for semi-quantitative RT-PCR. For real-time PCR, volumes were made up to 20 µl and contained SYBR Green 1 Buffer (QIAGEN, Hilden, Germany); forward and reverse primers used for β -actin detection were 5'-GACAGGATGCA-GAAGGAGATTACT-3' and 5'-TGATCCACATCTGCT-GGAAGGT-3' and NUR77 were 5'-GCTGCAGAATGAC TCCACC-3' and 5'-ACAGCAGCACTGGGCTTA-3'. Samples were run in duplicate with RNA preparations from three independent experiments. Real-time PCR was performed at 50 cycles (15 s at 95 °C, 56 s at 56 °C and 45 s at 68 °C), and each PCR run also included triplicate wells of no template control. A melting point dissociation curve generated by the instrument was used to confirm that only a single product was present. The fluorescence resulting from the incorporation of SYBR Green 1 dye into the double-stranded DNA produced during the PCR, and emission data were quantitated using the threshold cycle (C_t) value. Data were normalized to β -actin and presented as the mean fold change compared with control.

Statistical analysis

The effects of the agents on ICAM-1, VCAM-1 and PAI-1 protein production and mRNA expression were assessed by

Figure 1 (A) Dose response of liraglutide on 2-h TNF-stimulated induction of PAI-1 mRNA and protein secretion in C11 STH cells. C, control; T, TNF (10 ng/ml); TL, TNF + liraglutide; L, liraglutide ($n=3$; * $P<0.05$, T versus C, # $P<0.01$, T versus TL). (B) Effects of liraglutide treatment on basal and TNF-induced PAI-1 mRNA and protein secretion in C11 STH cells. (B (1)) Cells pretreated with TNF (10 ng/ml) for 5 h, and 100 nM liraglutide was added with TNF for 1 h subsequent to the 5-h incubation period. (B (2)) Cells pretreated with TNF (10 ng/ml) for 16 h, and 100 nM liraglutide was added with TNF for 1 h subsequent to the 16-h incubation period. C, control; T, TNF (10 ng/ml); TL, TNF + liraglutide; L, liraglutide ($n=3$, * $P<0.05$, T versus C, # $P<0.01$, T versus TL).

Figure 2 (A) Effects of liraglutide treatment on basal and TNF-induced ICAM-1 mRNA and protein secretion in C11 STH cells pretreated with TNF (10 ng/ml) for 5 h (1) or 16 h (2) and 100 nM liraglutide were added with TNF for 1 h subsequent to the 5- or 16-h incubation period. C, control; T, TNF; TL, TNF + liraglutide; L, liraglutide 100 nM ($n=3$; $*P<0.05$, T versus TL). (B) Effects of liraglutide treatment on basal and TNF-induced VCAM-1 mRNA and protein secretion in C11 STH cells pretreated with TNF (10 ng/ml) for 5 h (1) or 16 h (2) and 100 nM liraglutide were added with TNF for 1 h subsequent to the 5- or 16-h incubation period. C, control; T, TNF; TL, TNF + liraglutide; L, liraglutide 100 nM ($n=3$; $*P<0.05$, T versus TL).

ANOVA. Specific differences were tested with Bonferroni's *post hoc* comparison test. Data were expressed as mean $\pm$ S.E.M. and $P < 0.05$ was considered statistically significant.

Results

Liraglutide inhibits TNF-inducible PAI-1 protein expression in C11 STH cells

TNF treatment (10 ng/ml) resulted in a significant increase in PAI-1 mRNA and protein expression (Fig. 1A). Liraglutide dose response demonstrated that the addition of liraglutide at low concentrations (1 and 10 nM) demonstrated a non-significant trend towards the inhibition of TNF-mediated induction of PAI-1 expression. An addition of 100 nM or 1 μ M liraglutide significantly attenuated 2-h TNF-mediated induction of PAI-1 expression at 2 h to control levels (Fig. 1A). The 100 nM liraglutide treatment also significantly attenuated either 5- or 16-h TNF-mediated induction of PAI-1 mRNA and protein expression to control levels utilizing 5-h TNF pretreatment (Fig. 1B).

Liraglutide inhibits TNF-mediated ICAM-1 and VCAM-1 expression in C11 STH cells

Minimal constitutive expression of ICAM-1 or VCAM-1 was observed (Fig. 2A and B) in the C11 STH cell line. Liraglutide alone (100 nM) had no effect on constitutive ICAM-1 and VCAM-1 mRNA or protein expression (Fig. 2A and B). The 5- or 16-h TNF pretreatment (10 ng/ml) significantly induced ICAM-1 and VCAM-1 mRNA and protein expression (Fig. 2A and B). The 2-h TNF pretreatment resulted in no induction of ICAM-1 or VCAM-1 mRNA or protein expression (data not shown). Treatment with 100 nM liraglutide significantly attenuated 5- and 16-h pretreatment of TNF-mediated induction of ICAM-1 and VCAM-1 mRNA and protein expression (Fig. 2A and B).

Liraglutide inhibits hyperglycaemia-mediated PAI-1, ICAM-1 and VCAM-1 expression in C11 STH cells

The 10 mM glucose treatment significantly induced PAI-1, ICAM-1 and VCAM-1 mRNA and protein expression at

Figure 3 (A) Effects of liraglutide on hyperglycaemia-induced PAI-1 mRNA and protein secretion in C11 STH cells, C, control; L, liraglutide 100 nM; G10, 10 mM glucose; G10L, 10 mM glucose + liraglutide; ($n=3$; $*P < 0.05$, G10 versus C; $\#P < 0.01$, G10 vs G10L). (B) Effects of liraglutide on hyperglycaemia-induced ICAM-1 mRNA and protein secretion in C11 STH cells, C, control; L, liraglutide 100 nM; G10, 10 mM glucose; G10L, 10 mM glucose + liraglutide; ($n=3$; $*P < 0.05$, G10 versus C; $\#P < 0.01$, G10 vs G10L). (C) Effects of liraglutide on high-glucose-induced VCAM-1 mRNA and protein secretion in C11 STH cells, C, control; L, liraglutide 100 nM; G10, 10 mM glucose; G10L, 10 mM glucose + liraglutide; ($n=3$; $*P < 0.05$, G10 versus C; $\#P < 0.01$, G10 vs G10L).

48 h (Fig. 3A, B and C). Treatment of C11 STH cells with 100 nM liraglutide alone had no effect on the constitutive PAI-1, ICAM-1 and VCAM-1 expression (Fig. 3A, B and C). Treatment with 100 nM liraglutide significantly attenuated 10 mM glucose-mediated induction of PAI-1, ICAM-1 and VCAM-1 mRNA and protein expression at 48 h to control levels (Fig. 3A, B and C). A similar inhibitory response to 20 mM glucose induction of ICAM-1 and VCAM-1 by 100 nM liraglutide was also observed (data not shown).

Liraglutide inhibits TNF and hyperglycaemia-mediated expression of the orphan nuclear receptor NUR77 in C11 STH cells

The 5- or 16-h TNF pretreatment (10 ng/ml) or 10 mM glucose for 48 h significantly induced *Nur77* mRNA expression (Fig. 4A, B and C). Liraglutide alone (100 nM) had no effect on basal *Nur77* mRNA expression (Fig. 4A, B and C). Liraglutide treatment (100 nM) significantly attenuated TNF and glucose-mediated induction of *Nur77* mRNA expression to control levels (Fig. 4A, B and C).

Discussion

Increased expression of PAI-1 and vascular adhesion molecules have been observed in vascular endothelial cells stimulated by TNF or high glucose (Morigi *et al.* 1998, Sobel *et al.* 1998, Blankenberg *et al.* 2003, Liu *et al.* 2005, 2008, Norata *et al.* 2006, Kumar *et al.* 2007, Iwasaki *et al.* 2008), and are present in the plasma of patients with type 2 diabetes (Juhan-Vague *et al.* 1991, Steiner *et al.* 1994, Marfella *et al.* 2000, Peschel & Niebauer 2003). Elevated levels of PAI-1 and vascular adhesion molecules are associated with vascular endothelial cell dysfunction and may predispose patients to accelerated atherogenesis in the metabolic syndrome and type 2 diabetes (Juhan-Vague *et al.* 1991, Steiner *et al.* 1994, Marfella *et al.* 2000, Peschel & Niebauer 2003).

Previously we demonstrated that GLP-1 attenuates TNF-mediated induction of PAI-1 expression (Liu *et al.* 2008) and have been interested in the effects of liraglutide, an acylated long-acting GLP-1 analogue, on TNF- or high-glucose-induced PAI-1 and vascular adhesion molecule gene and protein expression in human vascular endothelial cells. We demonstrate for the first time, in a human cell system, that liraglutide attenuates TNF and high-glucose-induced PAI-1, ICAM-1 and VCAM-1 gene and protein expression in vascular endothelial cells.

TNF or high glucose activates transcription of adhesion molecules including ICAM-1 and VCAM-1 via translocation of NFκB from the cytoplasm into the cell nucleus with subsequent induction of gene expression (Morigi *et al.* 1998, Norata *et al.* 2006). TNF activates transcription of PAI-1 via modulation of NUR77 expression and binding to the NBRE-binding site in the PAI-1 gene promoter (Gruber *et al.* 2003, Liu *et al.* 2005, 2008). In addition, GLP-1-mediated inhibition

Figure 4 Real-time Q-PCR of the effects of liraglutide treatment on *Nur77* mRNA expression. C, control; T, TNF (10 ng/ml); G10, 10 mM glucose; L, liraglutide 100 nM; G10L, 10 mM glucose + liraglutide; TL, TNF + liraglutide; (*n* = 3; **P* < 0.05, G10 or T versus C; #*P* < 0.01, G10 or T versus G10L or TL). (A) 5-h TNF treatment, (B) 16-h TNF treatment, and (C) 48-h 10 mM glucose treatment.

of TNF-induced PAI-1 expression is associated with the suppression of NUR77 expression (Liu *et al.* 2008). Recent studies also demonstrate that high glucose induces PAI-1 expression through Rho/Rho-kinase-mediated NFκB activation in bovine aortic endothelial cells (Iwasaki *et al.* 2008). Also, GLP-1 together with glucose has been reported to modulate expression of NUR77 in the pancreatic β cell line, INS-1 (Susini *et al.* 1998).

To investigate the molecular mechanisms responsible for liraglutide-mediated inhibition of TNF- or high-glucose-induced PAI-1, ICAM-1 and VCAM-1 gene and protein expression, we explored the effects of liraglutide on *Nur77* mRNA expression. Our results demonstrate that the liraglutide treatment decreases TNF- and high-glucose-mediated

induction of *Nur77* mRNA expression, which is consistent with a possible molecular mechanism responsible for liraglutide's inhibition of elevated PAI-1, ICAM-1 and VCAM-1 expression under conditions of TNF and high-glucose stimulation.

Whilst the PAI-1 promoter has a recognized *cis*-acting element for NUR77 (NBRE; Gruber *et al.* 2003), it is currently unclear how liraglutide may regulate expression of ICAM-1 and VCAM-1 genes. It is of interest, however, that both ICAM-1 and VCAM-1 gene promoters harbour NUR77 *cis*-acting elements, which could mediate this effect (www.genomatrix.de/cgi-bin/matinspector).

Previous studies have demonstrated the involvement of NFκB in TNF- or high-glucose-induced PAI-1, ICAM-1, VCAM-1 expression (Morigi *et al.* 1998, Norata *et al.* 2006, Iwasaki *et al.* 2008). Interestingly, the NUR77 promoter harbours NFκB *cis*-acting regulatory elements (Pei *et al.* 2005), and the effects of liraglutide on this *trans*-acting factor and subsequent NUR77-mediated inhibition of induction of PAI-1, ICAM-1 and VCAM-1 warrant further investigation.

In summary, this *in vitro* study is the first to demonstrate liraglutide-mediated attenuation of TNF or high-glucose-mediated induction of PAI-1, ICAM-1 and VCAM-1 expression in human vascular endothelial cells. A postulated molecular mechanism responsible for this effect may involve regulation of NUR77 expression. These results indicate a potential role for liraglutide in the inhibition of endothelial cell dysfunction in type 2 diabetes and the metabolic syndrome. In addition, they complement recent *in vivo* studies demonstrating the beneficial effects of GLP-1 on endothelial cell dysfunction in diabetic patients (Nyström *et al.* 2004), which may, subject to the results of future studies, translate into improved cardiovascular outcomes in this patient population.

Declaration of interest

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