

Glucagon-like peptide-1 attenuates tumour necrosis factor- α -mediated induction of plasminogen activator inhibitor-1 expression

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Abstract

Glucagon-like peptide-1 (GLP-1) has been proposed as a target for treatment of type 2 diabetes. GLP-1 has also been demonstrated to improve endothelial cell dysfunction in diabetic patients. Elevated plasminogen activator inhibitor-1 (PAI-1) levels have been implicated in endothelial cell dysfunction. The effect of GLP-1 on PAI-1 expression in vascular endothelial cells has not been explored. In a spontaneously transformed human umbilical vein endothelial cell (HUVEC) line, C11-spontaneously transformed HUVEC (STH) and primary HUVEC cells, GLP-1 treatment, in the presence of a dipeptidyl peptidase IV inhibitor, attenuated induction of PAI-1 protein and mRNA expression by tumour necrosis factor- α (TNF- α). GLP-1 also

inhibited the effect of TNF- α on a reporter gene construct harbouring the proximal PAI-1 promoter. In addition, GLP-1 attenuated TNF- α -mediated induction of Nur77 mRNA and TNF- α -mediated binding of nuclear proteins (NPs) to the PAI-1, Nur77, *cis*-acting response element nerve growth factor induced clone B response element (NBRE). GLP-1 treatment also inhibited TNF- α -mediated induction of Akt phosphorylation. Taken together, these observations suggest that GLP-1 inhibits TNF- α -mediated PAI-1 induction in vascular endothelial cells, and this effect may involve Akt-mediated signalling events and the modulation of Nur77 expression and NP binding to the PAI-1 NBRE.

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Introduction

Glucagon-like peptide-1 (GLP-1), an incretin first identified in 1984, has been proposed as a potential candidate target for therapy in the treatment of type 2 diabetes (Nauck *et al.* 1993, Edwards 2005).

GLP-1, the product of the pre-proglucagon gene, is released from L cells in the intestine upon food intake and potently releases insulin from pancreatic β -cells in a glucose-dependent manner (Gromada *et al.* 1998, Vilsboll *et al.* 2001, Drucker & Nauck 2006). Interestingly, a number of additional effects of GLP-1 on the pancreas have been observed including stimulation of insulin biosynthesis and growth of β -cell mass (Drucker *et al.* 1987, Egan *et al.* 2003, Drucker & Nauck 2006). In the gut, GLP-1 inhibits motility and gastric emptying resulting in decreased food intake and reduced body weight (Wettergren *et al.* 1993, Flint *et al.* 1998, Knudsen 2004). Thus, GLP-1 may have a role in both glycaemic treatment of type 2 diabetes and potentially in the prevention or delay of disease progression.

In vivo, the half-life of native GLP-1 is estimated to be <2 min as a result of rapid renal clearance and degradation by the enzyme dipeptidyl peptidase IV (DPP-IV; Knudsen 2004). The poor bioavailability of GLP-1 mandates that for therapeutic usage the half-life of the molecule be enhanced. Homologues of GLP-1, including exenatide and liraglutide (Knudsen 2004), have been

developed, which have significantly prolonged half-lives when compared with the native peptide. DPP-IV inhibitors are also in an advanced stage of clinical development and early clinical use (Demuth *et al.* 2005, Drucker & Nauck 2006).

The biological activity of GLP-1 is thought to be mediated via a G-protein-linked receptor expressed on β -cells (Thorens 1992) and, among other cell types, vascular endothelial cells (Nyström *et al.* 2004). The presence of GLP-1 receptors on vascular endothelial cells, and evidence that GLP-1 can improve endothelial cell dysfunction (Richter *et al.* 1993, Yu *et al.* 2003, Nyström *et al.* 2004), suggests that GLP-1 may modulate vascular endothelial cell function. The effect of GLP-1 on PAI-1, implicated in endothelial cell dysfunction (Norata *et al.* 2007) and a regulator of plasminogen activation (Nicholl *et al.* 2006), in vascular endothelial cells has not yet been explored. Our study aimed to characterize the effects of GLP-1 on the regulation of tumour necrosis factor- α (TNF- α)-induced PAI-1 expression in vascular endothelial cells and to identify potential molecular mechanisms responsible for this effect.

Materials and Methods

Materials and cell culture

GLP-1 (truncated GLP-1 (7–36) amide) was purchased from Sigma, and the inhibitor of DPP-IV (valine pyrrolidide) was

provided by Novo Nordisk, Bagsvard, Denmark. The spontaneously transformed human umbilical vein endothelial cell (HUVEC), C11-STH, cell line (Cockerill *et al.* 1994) and primary HUVEC were cultured under sterile conditions. Experiments were performed at between three and four cell passages (primary HUVEC) and 80% cellular confluence at 37 °C using gelatin-coated Nunclon cell culture dishes in Media-199 (Sigma) supplemented with penicillin/streptomycin, 20% fetal calf serum (FCS), 20 µg/ml endothelial cell growth factor (Sigma) and 20 µg/ml heparin. Glucose concentration in media was 6.0 mmol/l.

Determination of PAI-1 protein

PAI-1 protein was determined in C11-STH and HUVEC cells under varying conditions. Cells were incubated under serum-free conditions with 10 ng/ml TNF- α (Promega) for 5 h or overnight (18 h). GLP-1 (0.03–30 nM) and/or DPP-IV inhibitor (20 µM) were added for 1 h either prior or subsequent to the incubation period with TNF- α . Where not stated, for all the other experiments, cells were incubated for 5 h with TNF- α and then GLP-1 and DPP-IV inhibitor added subsequently for 1 h.

The conditioned medium (CM) was subsequently collected and centrifuged at 8161 g for 1 min, the PAI-1 protein concentration in the CM was assayed using a PAI-1 ELISA (Chromolize PAI-1 Trinity Biotech plc, Bray, Ireland). One unit of PAI-1 activity is defined as the amount of PAI-1 that inhibits one international unit of human single chain tissue type plasminogen activator (tPA) as calibrated against the international standard for tPA, lot 86/670 distributed by NIBSC, Holly Hill, London, England. The Chromolize PAI-1 ELISA is also standardized against the NIBSC PAI-1 standard 92/654. Coefficient of variation (CV)% within-run in this kit is <3.7%, CV% run–run is <4.6%. The detection range is 2.0–50 IU/ml. All experiments were performed with triplicate incubates and repeated independently three times.

Northern blot procedure

Total RNA was extracted from C11-STH cells by the method of Chomczynski & Sacchi (1987). Ten micrograms of RNA were loaded on to each lane and electrophoresed through a 1% agarose gel containing 20% formaldehyde before being transferred to Hybond-N⁺ membrane (Amersham). Filters were hybridized overnight at 42 °C in 50% formamide hybridization buffer. ³²P-labelling of DNA was performed by the random priming procedure using the Prima-gene labelling kit (Promega). Hybridization using the Pst-1 fragments of PAI-1 was performed (Liu *et al.* 2005). After hybridization, the membranes were washed by standard techniques and exposed to Kodak BioMax film (Eastman Kodak) at 70 °C with an intensifying screen. All northern blot experiments were repeated independently three times.

Real-time quantitative PCR

RNA (extracted as for northern blot) was heated to 65 °C for 10 min. Subsequently, reverse transcriptase (Invitrogen) together with oligo (dT)₁₅ primers in the presence of 1 mM dNTP and dithiothreitol (DTT) was added in order to generate the first-strand cDNA. For real-time PCR, volumes were made up to 20 µl and contained SYBR Green 1 Buffer (Eppendorf, Hamburg, Germany), and forward and reverse primers for β -actin are 5'-GACAGGATGCAGAAGGA-GATTACT-3' and 5'-TGATCCACATCTGCTGGAAG-GT-3' respectively. Forward and reverse primers for nur77 are 5'-GCTGCAGAATGACTCCACC-3' and 5'-ACAGC-AGCACTGG GCTTA-3' respectively. Samples were run in duplicate with RNA preparations from three independent experiments. Real-time PCR was performed at 50 cycles (95 °C 15 s, 56 °C 56 s and 68 °C 45 s), 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 when compared with control.

Transient transfection and chloramphenicol acetyl transferase (CAT) assay

PAI-1-CAT promoter construct containing the first 1.4 kb human PAI-1 gene promoter sequence, previously constructed in the laboratory (Liu *et al.* 2005), was transiently transfected using the calcium phosphate precipitation method (Costa *et al.* 1998) into C11-STH cells. Cells were also co-transfected with plasmid pSV- β -galactosidase (Promega) and β -galactosidase activity used as an internal control for transfection efficiency. Transfected cells were harvested and cellular extracts prepared. CAT activity assay was performed by incubating 40 µl protein extracts (25 µg protein), with 4 µl of 4.4 mM acetyl CoA and 1.0 µl ¹⁴C-chloramphenicol at 37 °C for 4 h. Samples were extracted with 1 ml ethyl acetate, vacuum dried and finally dissolved in 20 µl ethyl acetate. The samples were spotted onto thin layer chromatography (TLC) plates and processed using standard techniques. Conversion of ¹⁴C-chloramphenicol into its acetylated product was assessed by autoradiography. β -Galactosidase activity in cell extracts was determined. CAT activity was normalized by adjusting for changes in β -galactosidase activity in the same samples (Costa *et al.* 1998). All experiments were performed with triplicate incubates and repeated independently three times.

Preparation of nuclear extracts

C11-STH cells were lysed in NP40 lysis buffer (0.5% NP40, 10 mM NaCl, 10 mM Tris (pH 7.4), 3 mM MgCl₂, 5 mM

DTT and 1 mM phenylmethylsulphonyl fluoride (PMSF); Costa *et al.* 1998). After 10-min incubation on ice, samples were briefly centrifuged and washed with 0.2 ml NP40 buffer, pellets were resuspended in 100 µl buffer C (420 mM NaCl, 20 mM HEPES (pH 7.9), 1.5 mM MgCl₂, 0.2 mM EDTA, 25% glycerol, 5 mM DTT and 5 mM PMSF; Costa *et al.* 1998). After incubation on ice for 20 min and centrifugation at 13 000 *g* at 4 °C for 20 min, the supernatants containing nuclear proteins (NPs) were collected and stored at -80 °C.

Electrophoretic mobility shift assay (EMSA)

Gel-purified oligonucleotides (100 ng) were labelled using T4 polynucleotide kinase and γ -³²P ATP. Annealing was performed by adding a fourfold molar excess of the complementary strand. After elution from native acrylamide gels, samples were precipitated with ethanol and finally resuspended in NaCl/Tris/EDTA buffer (100 mM NaCl, 100 mM Tris (pH 7.4) and 10 mM EDTA (pH 7.4)).

To perform the EMSAs, 4–10 µg NP extracts in 4 µl Osborne buffer D (Osborne *et al.* 1989) were incubated at 4 °C for 15 min with 1 µl poly d[I-C] (Boehringer Mannheim) and 3 µl SMK buffer (12 mM spermidine, 12 mM MgCl₂ and 200 mM KCl). Four microlitres of ³²P-labelled probe (100 c.p.m.; NBRE oligonucleotide sequence was 5'-AGA GGG CAG AAA GGT CAA GGG-3') diluted in Osborne buffer D were then added and the mixtures incubated on ice for a further 15 min before being applied to a native 5% polyacrylamide gel.

For competition experiments to demonstrate specificity of NP binding, extracts were incubated with 100 ng unlabelled oligomers of identical (5'-AGA GGG CAG AAA GGT CAA GGG-3') or scrambled (5'-TCA GCA AGG GAA ATG GCT T-3') sequence for 15 min prior to the addition of the probe. After electrophoresis, gels were fixed, dried and autoradiographed overnight at 70 °C with an intensifying screen. All EMSAs were repeated independently three times.

Immunoblotting

C11-STH cells were collected by centrifugation (13793 *g* for 1 min), resuspended in 400 µl cold buffer A (10 mmol/l HEPES (pH 7.9), 10 mmol/l KCl, 0.1 mmol/l EDTA, 0.1 mmol/l EGTA, 1 mmol/l DTT and a protease inhibitor mixture (Roche Diagnostics) and incubated on ice for 15 min. The homogenates were centrifuged (13793 *g* for 1 min) and supernatants (cytosolic fraction) stored at -80 °C. Protein concentration was determined by the bicinchoninic acid protein assay (Pierce, Rockford, IL, USA). Samples were run on 10% SDS-PAGE gel and transferred to polyvinylidenedifluoride (PVDF) membrane (Millipore, Billerica, MA, USA). Membranes were incubated at 4 °C overnight with anti-Akt and anti-phospho-Ser-473 Akt (Cell Signalling, Rockford, IL, USA). Immunoreactive bands were detected by chemiluminescence with enhanced chemiluminescence plus reagents (Amersham). All immunoblot experiments were repeated independently three times.

Scanning densitometry

Scanning densitometry analyses were performed on northern blots and CAT assay using the ImageQuant 5.2 software (Molecular Dynamics, Sunnyvale, USA). Scanning densitometry analyses were performed on western blots using the GeneSnap imaging software (SYNOPTICS, Cambridge, UK).

Statistical methods

The effects of agents on PAI-1 protein production, mRNA expression and CAT activity were assessed by ANOVA. Specific differences were tested with Bonferroni's *post hoc* comparison test. Data were expressed as means \pm s.e.m., and *P* < 0.05 was considered statistically significant.

Results

Effect of GLP-1 on TNF- α -inducible PAI-1 protein expression

PAI-1 protein expression in C11-STH cells and primary HUVEC was significantly induced by a concentration of 10 ng/ml TNF- α , previously documented to induce PAI-1 expression (Norata *et al.* 2006; Fig. 1).

Dose-response demonstrated that addition of GLP-1, in the absence of the DPP-IV inhibitor, at various concentrations did not inhibit TNF- α -mediated induction of PAI-1 expression, although a non-significant trend towards inhibition of PAI-1 expression was observed (Fig. 1A).

To determine the effects of GLP-1 at a concentration previously determined to be within the physiological range (Vilsboll *et al.* 2003) on TNF- α -mediated induction of PAI-1 expression, 0.3 nM GLP-1 together with the DPP-IV inhibitor (20 µM) was used in C11-STH cells. GLP-1 and/or DPP-IV inhibitor were added for 1 h subsequent to the 5-h incubation period with TNF- α (Fig. 1B (1)) or 1-h pre-treatment with GLP-1 and/or DPP-IV inhibitor and then 5-h incubation period with TNF- α (Fig. 1B (2)). The combination of 0.3 nM GLP-1 and DPP-IV inhibitor, pre- or post-TNF- α stimulation, resulted in significant inhibition of TNF- α -mediated induction of PAI-1 expression (Fig. 1B (1) and (2)). The inhibitor of DPP-IV or GLP-1 (0.3 nM) alone, or in combination, had no effect on basal PAI-1 expression (Fig. 1B (1) and (2)). No significant inhibitory effect of DPP-IV inhibitor or GLP-1 alone on TNF- α -mediated induction of PAI-1 expression (Fig. 1B (1) and (2)) was observed.

Primary HUVEC cells incubated with GLP-1 (0.3 nM) and/or DPP-IV inhibitor (20 µM) for 1 h subsequent to the 5-h incubation period with TNF- α also demonstrated significant inhibition of TNF- α -mediated induction of PAI-1 protein expression (Fig. 1C).

Pre-treatment of C11-STH cells with GLP-1 and DPP-IV inhibitor for 1 h prior to overnight TNF- α stimulation demonstrated significant inhibition of TNF- α -mediated induction of PAI-1 expression (Fig. 1D (2)). However, when GLP-1 and DPP-IV inhibitor were added for 1 h

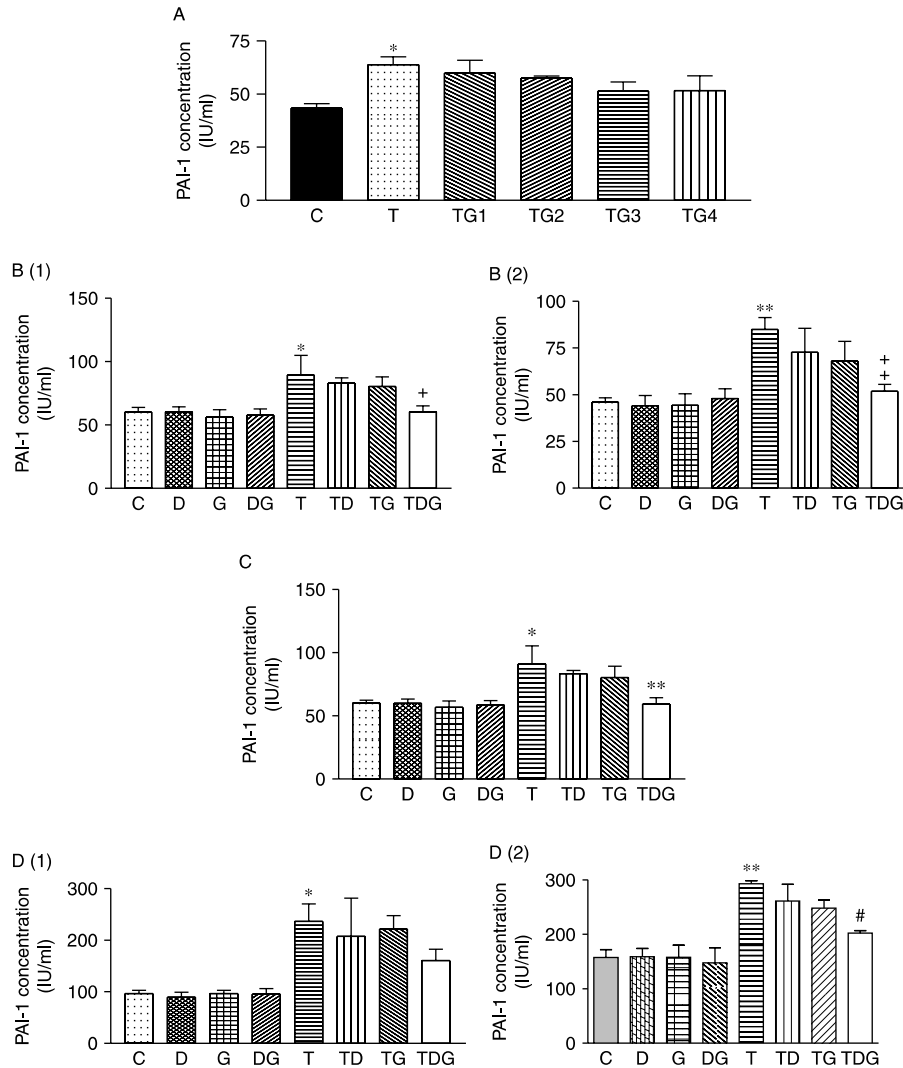


Figure 1 (A) Dose-response of GLP-1 on TNF- α -induced PAI-1 protein secretion in C11-STH cells. C, Control; T, TNF- α ; TG1, TNF- α + 0.03 nM GLP-1; TG2, TNF- α + 0.3 nM GLP-1; TG3, TNF- α + 3.0 nM GLP-1; TG4, TNF- α + 30.0 nM GLP-1 ($n=3$; * $P<0.05$, T versus C). (B) Effects of GLP-1 and/or the inhibitor of DPP-IV treatment on basal and TNF- α -induced PAI-1 protein secretion in C11-STH cells. B (1) cells were pre-treated with TNF- α 10 ng/ml for 5 h. GLP-1 (0.3 nM) and/or DPP-IV inhibitor (20 μ M) were added for 1 h subsequent to the 5-h incubation period with TNF- α . B (2) cells were pre-treated with GLP-1 (0.3 nM) and/or DPP-IV inhibitor (20 μ M) for 1 h before the 5-h incubation period with TNF- α . C, control; T, TNF- α ; D, DPP-IV inhibitor (20 μ M); G, GLP-1 (0.3 nM); DG, DPP-IV inhibitor (20 μ M) + GLP-1 (0.3 nM); TD, TNF- α + DPP-IV inhibitor (20 μ M); TG, TNF- α + GLP-1 (0.3 nM); TDG, TNF- α + DPP-IV inhibitor (20 μ M) + GLP-1 (0.3 nM) ($n=3$; * $P<0.05$, T versus C; + $P<0.05$, TDG versus T, ** $P<0.01$, T versus C, ++ $P<0.01$, TDG versus T). (C) Effects of GLP-1 and/or the inhibitor of DPP-IV treatment on basal and TNF- α -induced PAI-1 protein secretion in primary HUVEC cells. C, control; T, TNF- α ; D, DPP-IV inhibitor (20 μ M); G, GLP-1 (0.3 nM); DG, DPP-IV inhibitor (20 μ M) + GLP-1 (0.3 nM); TD, TNF- α + DPP-IV inhibitor (20 μ M); TG, TNF- α + GLP-1 (0.3 nM); TDG, TNF- α + DPP-IV inhibitor (20 μ M) + GLP-1 (0.3 nM) ($n=3$; * $P<0.01$, T versus C; ** $P<0.01$, TDG versus T). (D) Effects of GLP-1 and/or the inhibitor of DPP-IV treatment on basal and TNF- α -induced PAI-1 protein secretion in C11-STH cells. D (1) cells were pre-treated with TNF- α 10 ng/ml for overnight. GLP-1 (0.3 nM) and/or DPP-IV inhibitor (20 μ M) were added for 1 h subsequent to the overnight incubation period with TNF- α . D (2) cells were pre-treated with GLP-1 (0.3 nM) and/or DPP-IV inhibitor (20 μ M) for 1 h before the overnight incubation period with TNF- α . C, control; T, TNF- α ; D, DPP-IV inhibitor (20 μ M); G, GLP-1 (0.3 nM); DG, DPP-IV inhibitor (20 μ M) + GLP-1 (0.3 nM); TD, TNF- α + DPP-IV inhibitor (20 μ M); TG, TNF- α + GLP-1 (0.3 nM); TDG, TNF- α + DPP-IV inhibitor (20 μ M) + GLP-1 (0.3 nM) ($n=3$; * $P<0.01$, T versus C; ** $P<0.001$, T versus C, # $P<0.05$, TDG versus T).

subsequent to overnight incubation with TNF- α , a non-significant inhibitory effect of DPP-IV inhibitor plus GLP-1 on TNF- α -mediated induction of PAI-1 expression (Fig. 1D (1)) was observed. No significant inhibitory effect of DPP-IV inhibitor or GLP-1 alone or in combination on basal or TNF- α -mediated induction of PAI-1 expression (Fig. 1D (1) and D (2)) was observed.

Effect of GLP-1 on TNF- α -inducible PAI-1 mRNA expression in C11-STH cells

Treatment of C11-STH cells with 0.3 nM GLP-1 or the DPP-IV inhibitor (20 μ M) alone or in combination for 1 h had no effect on constitutive mRNA PAI-1 expression (Fig. 2A). TNF- α treatment (10 ng/ml) for 5 h significantly induced PAI-1 mRNA expression (Fig. 2B). Treatment with GLP-1 (0.3 nM) together with the inhibitor of DPP-IV, for 1 h subsequent to TNF- α stimulation, significantly attenuated TNF- α -mediated induction of PAI-1 mRNA expression (Fig. 2B). No significant inhibitory effect of DPP-IV

inhibitor or GLP-1 alone on TNF- α -mediated induction of PAI-1 expression (Fig. 2B) was observed.

Both the upper (3.2 kb) and lower (2.3 kb) PAI-1 mRNA transcripts were identified as described previously (Loskutoff *et al.* 1987). Quantification of both PAI-1 mRNA transcripts was performed in all northern blot experiments to facilitate graphical data presentation (Fig. 2A(ii) and B(ii)).

Effects of GLP-1 on PAI-1 promoter reporter gene activity in C11-STH cells

In C11-STH cells co-transfected with the PAI-1-CAT construct and plasmid pSV- β -galactosidase, treatment with TNF- α , for 5 h, significantly increased expression of the 1.4 kb PAI-1-CAT expression vector (Fig. 3). GLP-1 (0.3 nM) or DPP-IV inhibitor treatment alone, for 1 h, had no effect on basal or TNF- α -mediated CAT activity. GLP-1 treatment together with the inhibitor of DPP-IV significantly attenuated TNF- α -inducible CAT activity (Fig. 3) suggesting a transcriptionally mediated effect.

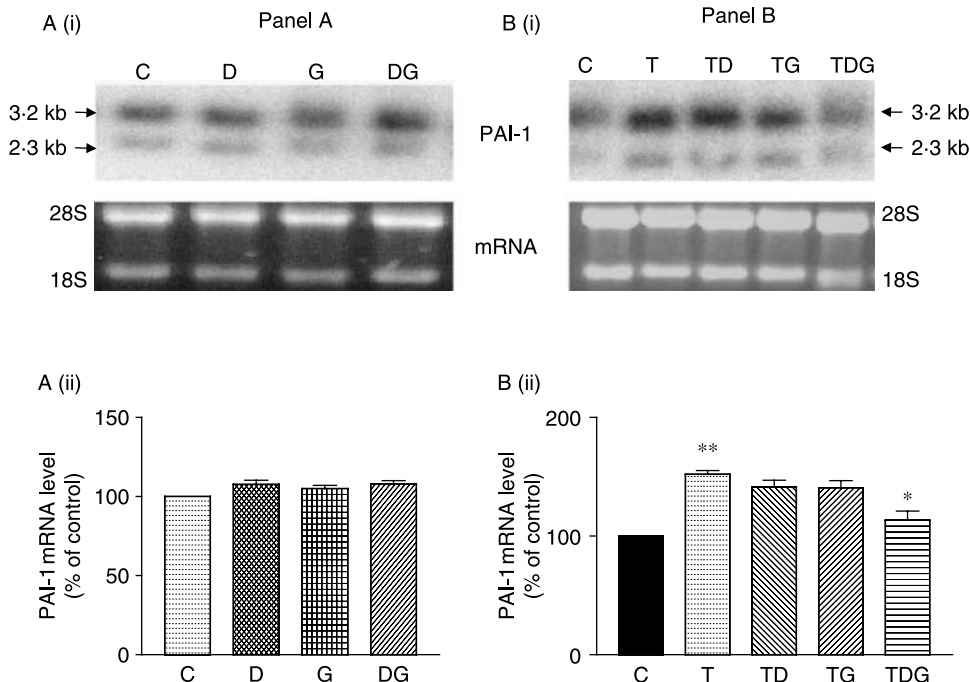


Figure 2 A (i) Northern blot of effect of GLP-1 and the inhibitor of DPP-IV treatment on constitutive PAI-1 mRNA expression in C11-STH cells. 18S and 28S mRNA expression provides a loading control. Arrow heads point to 3.2 and 2.3 kb PAI-1 transcripts. A (ii) Quantification of GLP-1 and the inhibitor of DPP-IV treatment on constitutive PAI-1 mRNA expression. B (i) Northern blot of GLP-1 together with the inhibitor of DPP-IV treatment on TNF- α -induced PAI-1 mRNA expression in C11-STH cells. 18S and 28S mRNA expression provides a loading control. Arrow heads point to 3.2 and 2.3 kb PAI-1 transcripts. B (ii) Quantification of GLP-1 together with the inhibitor of DPP-IV treatment on TNF- α -induced PAI-1 mRNA expression. C, control; T, TNF- α ; D, DPP-IV inhibitor (20 μ M); G, GLP-1 (0.3 nM); DG, DPP-IV inhibitor (20 μ M)+GLP-1 (0.3 nM); TD, TNF- α +DPP-IV inhibitor (20 μ M); TG, TNF- α +GLP-1 (0.3 nM); TDG, TNF- α +DPP-IV inhibitor (20 μ M)+GLP-1 (0.3 nM) ($n=3$; ** $P<0.05$, T versus C; * $P<0.05$, TDG versus T).

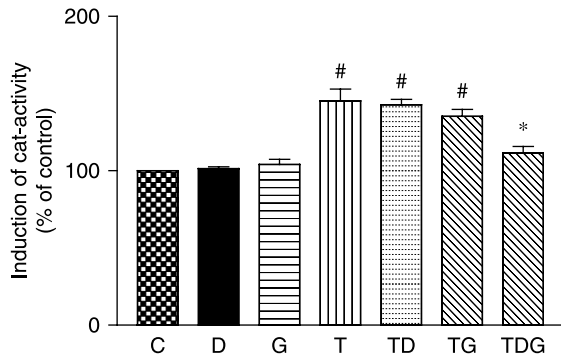


Figure 3 Effect of GLP-1 and the inhibitor of DPP-IV treatment on PAI-1-CAT reporter gene activity in C11-STH cells. C, control; T, TNF- α ; D, DPP-IV inhibitor (20 μ M); G, GLP-1 (0.3 nM); TD, TNF- α + DPP-IV inhibitor (20 μ M); TG, TNF- α + GLP-1 (0.3 nM); TDG, TNF- α + DPP-IV inhibitor (20 μ M) + GLP-1 (0.3 nM) ($n=3$; # $P<0.001$, T, TD and TG versus C; * $P<0.05$; TDG versus T).

Effect of GLP-1 on orphan nuclear receptor Nur77 expression and NP binding to the PAI-1 NBRE in C11-STH cells

TNF- α treatment (10 ng/ml; 5 h) significantly induced mRNA expression of the orphan nuclear receptor Nur77 (Fig. 4). Treatment with GLP-1 (0.3 nM) or the inhibitor of DPP-IV (20 μ M), alone or in combination for 1 h, had no significant effect on constitutive Nur77 mRNA expression (Fig. 4) in C11-STH vascular endothelial cells. Treatment with GLP-1 together with the inhibitor of DPP-IV for 1 h subsequent to 5-h TNF- α stimulation significantly attenuated TNF- α -mediated induction of Nur77 mRNA expression (Fig. 4), while treatment of TNF- α -stimulated cells with either GLP-1 or DPP-IV inhibitor alone had no significant

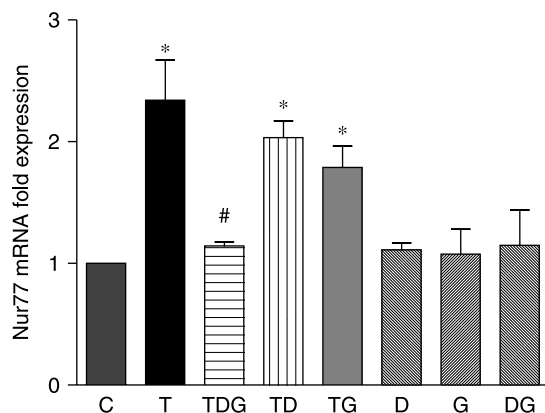


Figure 4 Real-time quantitative PCR of the effect of GLP-1 and the inhibitor of DPP-IV treatment on Nur77 mRNA expression in C11-STH cells. C, control; T, TNF- α ; D, DPP-IV inhibitor (20 μ M); G, GLP-1 (0.3 nM); DG, DPP-IV inhibitor (20 μ M) + GLP-1 (0.3 nM); TD, TNF- α + DPP-IV inhibitor (20 μ M); TG, TNF- α + GLP-1 (0.3 nM); TDG, TNF- α + DPP-IV inhibitor (20 μ M) + GLP-1 (0.3 nM) ($n=3$; * $P<0.001$, T, TD and TG versus C; # $P<0.01$, TDG versus T).

inhibitory effect on TNF- α -mediated induction of Nur77 expression.

NPs extracted from C11-STH cells specifically bound to a 32 P-labelled oligonucleotide harbouring the PAI-1 NBRE element as demonstrated by EMSA. Increased binding of NPs to the PAI-1 NBRE was observed upon TNF- α stimulation for 5 h. Treatment with GLP-1 together with the inhibitor of DPP-IV for 1 h, in the absence of TNF- α stimulation, had no effect on binding of NPs to the PAI-1 NBRE. Treatment with GLP-1 (0.30 nM) or the inhibitor of DPP-IV (20 μ M) for 1 h alone had no effect on TNF- α -mediated induction of NP binding, while treatment with GLP-1 together with the inhibitor of DPP-IV for 1 h subsequent to 5-h TNF- α stimulation attenuated the TNF- α -mediated increase in binding of NPs (Fig. 5).

Effects of GLP-1 on phosphorylation of Akt in C11-STH cells

GLP-1 has been reported to activate protein kinase B (PKB also known as Akt; Wang *et al.* 2004). Hence, we examined the effects of GLP-1 on activated (phospho-Akt) expression. In C11-STH cells treatment with TNF- α for 5 h significantly increased phosphorylation of Akt. Treatment with GLP-1 (0.30 nM) and/or the inhibitor of DPP-IV (20 μ M) for 1 h, in the absence of TNF- α stimulation, had no effect on phosphorylation of Akt. Treatment with GLP-1 or the inhibitor of DPP-IV alone for 1 h subsequent to treatment

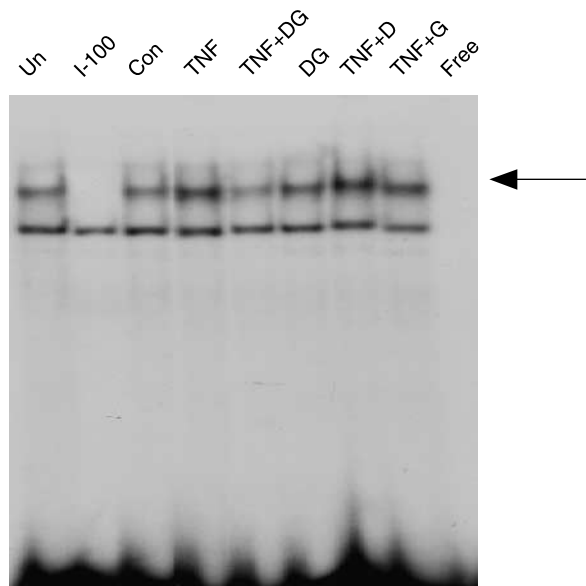


Figure 5 EMSA: nuclear extracts from C11-STH cells that were untreated (Con) and incubated with a 100-fold molar excess of unlabelled identical (I-100) or unrelated (Un) oligonucleotide. DG, DPP-IV inhibitor (20 μ M) + GLP-1 (0.3 nM) treated cells; TNF, TNF- α -treated cells; TNF + DG, TNF- α + DPP-IV inhibitor + GLP-1-treated cells; TNF + D, TNF- α + DPP-IV inhibitor-treated cells; TNF + G, TNF- α + GLP-1-treated cells; Free, Free probe, no nuclear extract. Specific binding to the PAI-1 NBRE is indicated by the black arrow.

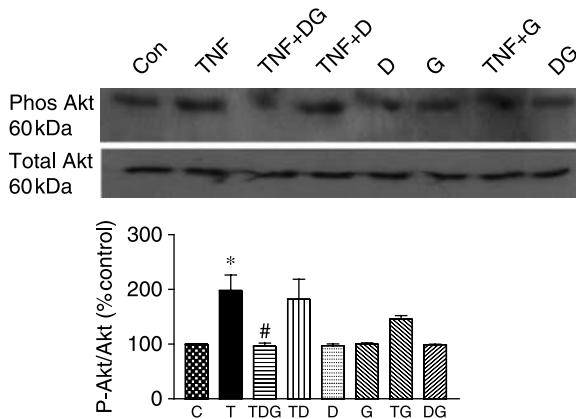


Figure 6 Effect of GLP-1 and the inhibitor of DPP-IV treatment on Akt phosphorylation in C11-STH cells. Con, control; TNF, TNF- α ; D, DPP-IV inhibitor (20 μ M); G, GLP-1 (0.3 nM); DG, DPP-IV inhibitor (20 μ M) + GLP-1 (0.3 nM); TNF+D, TNF- α + DPP-IV inhibitor (20 μ M); TNF+G, TNF- α + GLP-1 (0.3 nM); TNF+DG, TNF- α + DPP-IV inhibitor (20 μ M) + GLP-1 (0.3 nM). Histogram demonstrating quantification of western blot of the ratio of phospho-Akt to total Akt. C, control; T, TNF- α ; D, DPP-IV inhibitor (20 μ M); G, GLP-1 (0.3 nM); DG, DPP-IV inhibitor (20 μ M) + GLP-1 (0.3 nM); TD, TNF- α + DPP-IV inhibitor (20 μ M); TG, TNF- α + GLP-1 (0.3 nM); TDG, TNF- α + DPP-IV inhibitor (20 μ M) + GLP-1 (0.3 nM) ($n=3$; * $P<0.01$, T versus C; # $P<0.01$, TDG versus T).

with TNF- α for 5 h had no effect on TNF- α -mediated induction of phosphorylation of Akt while treatment with GLP-1 together with the inhibitor of DPP-IV significantly attenuated the TNF- α -mediated increase in phosphorylation of Akt (Fig. 6).

Discussion

We have demonstrated for the first time that GLP-1 attenuates TNF- α -induced PAI-1 gene and protein expression in a spontaneously transformed vascular endothelial cell line (C11-STH) and in primary human vascular endothelial cells. The dose-response observations suggest that GLP-1 treatment alone results in a trend towards inhibition of TNF- α -mediated induction of PAI-1 expression at concentrations as low as 0.30 nM (Fig. 1A). However, this trend was not significant, perhaps due to the endogenous cell-bound DPP-IV. The addition of a DPP-IV inhibitor to the cell culture to extend the known short $t_{1/2}$ of GLP-1 (Fig. 1B–D) enabled us to demonstrate an effect of GLP-1 in our endothelial model, with the most robust inhibition if PAI-1 expression observed at 1-h incubation with GLP-1 and DPP-IV inhibitor subsequent to 5-h stimulation with TNF- α . GLP-1 and DPP-IV inhibitor treatment, pre- or post-TNF- α stimulation, in the short term, inhibits TNF- α -mediated induction of PAI-1 expression (Fig. 1B(1) and B(2)), while overnight stimulation with TNF- α requires pre-incubation with GLP-1 together with DPP-IV inhibitor treatment to inhibit the significant TNF- α -mediated increase in PAI-1 expression (Fig. 1D (1) and D (2)).

Confirmation of our observations, which demonstrate remarkable consistency, using different treatment schedules and time points, including administration of GLP-1 and DPP-IV inhibitor, pre- and post-TNF- α stimulation, support the validity of our findings. Confirmation of our primary end point of GLP-1 and DPP-IV inhibitor-mediated inhibition of TNF- α -mediated induction of PAI-1 expression in two cell systems, primary HUVEC together with the C11-STH cell line, supports the contention that our observations reflect genuine vascular endothelial cell responses to GLP-1 and DPP-IV inhibitor treatment.

Identification of regulation of TNF- α -mediated induction of PAI-1 mRNA expression and of a PAI-CAT promoter construct by GLP-1 and the DPP-IV inhibitor suggests a transcriptionally mediated mechanism of action of GLP-1 and the DPP-IV inhibitor on PAI-1 expression, the molecular mechanism of which was subsequently explored.

To investigate the molecular mechanisms for GLP-1-mediated inhibition of TNF- α -induced PAI-1 expression, we explored the effects of GLP-1 on Nur77 mRNA expression together with the binding of NPs to the PAI-1, Nur77, *cis*-acting response element, NBRE. Binding of NPs including Nur77 to the PAI-1 NBRE has previously been identified as responsible for mediating induction of PAI-1 expression in response to TNF- α stimulation (Gruber *et al.* 2003). Our results demonstrated that GLP-1 treatment could decrease TNF- α -mediated Nur77 mRNA expression and NP binding to the PAI-1 NBRE, suggesting a molecular mechanism responsible for GLP-1 inhibition of elevated PAI-1 expression under conditions of TNF- α stimulation.

GLP-1 has been shown to regulate the activity of several *trans*-acting factors such as pancreatic/duodenal homeobox 1 (PDX1; Campbell & Macfarlane 2000) through modulation of the PDX1 gene promoter (Wang *et al.* 1999) and islet amyloid polypeptide, again through its gene promoter (Shepherd *et al.* 2004). In addition, GLP-1 has been shown to regulate the expression of a number of β -cell-specific genes, including insulin (Wang *et al.* 1995), glucose transporter type 2 (GLUT 2; Waeber *et al.* 1996) and glucokinase (Watada *et al.* 1996), and together with glucose, GLP-1 has also been reported to modulate expression of Nur77 in a pancreatic β -cell line, INS-1 (Susini *et al.* 1998). Our observations describe for the first time GLP-1-mediated modulation of Nur77 expression in vascular endothelial cells.

GLP-1-mediated Nur77 modulation of PAI-1 expression may involve growth factor-like signalling pathways (Trümper *et al.* 2000). Of particular importance is the ability of GLP-1 to activate PKB (also known as Akt; Wang *et al.* 2004). Akt activation results in the regulation of the DNA-binding activity and phosphorylation of Nur77 (Masuyama *et al.* 2001, Pekarsky *et al.* 2001). Our results demonstrate a possible intracytoplasmic signalling pathway for GLP-1-mediated gene regulation. GLP-1 treatment inhibits TNF- α -mediated stimulation of Akt phosphorylation which may in turn inhibit TNF- α -mediated PAI-1 induction via decreasing Nur77

mRNA and protein expression and/or inhibiting Nur77 DNA-binding activity to the PAI-1 NBRE.

In summary, this is the first report to demonstrate that GLP-1 attenuates TNF- α -mediated induction of PAI-1 expression in vascular endothelial cells. The mechanism responsible for this effect may occur through Akt-mediated regulation of Nur77 expression and/or DNA binding. Our *in vitro* observations complement recent *in vivo* studies demonstrating beneficial effects of GLP-1 on endothelial cell dysfunction in diabetic patients (Nyström *et al.* 2004). These observations may, subject to results of future studies, translate into improved cardiovascular outcomes in this vulnerable patient population.

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