Exendin-4 protects endothelial cells from lipoapoptosis by PKA, PI3K, eNOS, p38 MAPK, and JNK pathways

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Abstract

Experimental studies have indicated that endothelial cells play an important role in maintaining vascular homeostasis. We previously reported that human coronary artery endothelial cells (HCAECs) express the glucagon-like peptide 1 (GLP1) receptor and that the stable GLP1 mimetic exendin-4 is able to activate the receptor, leading to increased cell proliferation. Here, we have studied the effect of exendin-4 and native GLP1 (7–36) on lipoapoptosis and its underlying mechanisms in HCAECs. Apoptosis was assessed by DNA fragmentation and caspase-3 activation, after incubating cells with palmitate. Nitric oxide (NO) and reactive oxidative species (ROS) were analyzed. GLP1 receptor activation, PKA-, PI3K/Akt-, eNOS-, p38 MAPK-, and JNK-dependent pathways, and genetic silencing of transfection of eNOS were also studied. Palmitate-induced apoptosis stimulated cells to release NO and ROS, concomitant with upregulation of eNOS, which required activation of p38 MAPK and JNK. Exendin-4 restored the imbalance between NO and ROS production in which ROS production decreased and NO production was further augmented. Incubation with exendin-4 and GLP1 (7–36) protected HCAECs against lipoapoptosis, an effect that was blocked by PKA, PI3K/Akt, eNOS, p38 MAPK, and JNK inhibitors. Genetic silencing of eNOS also abolished the anti-apoptotic effect afforded by exendin-4. Our results support the notion that GLP1 receptor agonists restore eNOS-induced ROS production due to lipotoxicity and that such agonists protect against lipoapoptosis through PKA-PI3K/Akt-eNOS-p38 MAPK-JNK-dependent pathways via a GLP1 receptor-dependent mechanism.

Key Words

- ▶ Endothelial nitric oxide synthase
- ▶ Exendin-4
- ▶ GLP1
- ▶ Human coronary endothelial cells
- ▶ Lipoapoptosis

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Introduction

Type 2 diabetes and insulin resistance are associated with atherosclerosis, which is the major cause of morbidity and mortality in these patients. Experimental studies have indicated that the vascular endothelium plays an important role in maintaining the balance between vasoconstriction and vasodilatation, coagulation and fibrinolysis,

proliferation, and apoptosis (Furchgott & Vanhoutte 1989). Damage of vascular cells by proatherosclerotic risk factors, such as lipids and inflammatory cytokines, might be an early marker for the development of atherosclerosis and vascular complications (Verma *et al.* 2003). Several studies have reported that free fatty acids (FFAs) impair the

integrity of the endothelial monolayer and its barrier function, leading to enhanced endothelial apoptosis (Chai & Liu 2007, Artwohl *et al.* 2008), a key factor contributing to endothelial dysfunction (Dimmeler *et al.* 2002). Nitric oxide (NO) is the single most important factor for maintaining endothelial function, and mice with a deletion of the gene for endothelial NO synthase (eNOS) show accelerated atherosclerosis (Kuhlencordt *et al.* 2001). Circulating FFAs interfere with eNOS (Steinberg *et al.* 2000) and endothelial function in humans (Tripathy *et al.* 2003).

Glucagon-like peptide-1 (GLP1) is a brain gut insulinotropic hormone that plays an important role in the regulation of glucose homeostasis and thus has been applied in clinical treatment of diabetes (Drucker & Nauck 2006). Exendin-4, a stable incretin mimetic and GLP1 receptor agonist, has been shown to promote glucose-dependent insulin secretion *in vitro* (Parkes *et al.* 2001). Besides its insulinotropic action, exendin-4 may also exert direct beneficial effects on endothelial function. Exendin-4 reduces macrophage adhesion to the endothelium, an early step in the formation of atherosclerotic plaques and endothelial dysfunction (Arakawa *et al.* 2010). Acute administration of exendin-4 in humans prevents endothelial dysfunction induced by ischemia reperfusion injury (Ha *et al.* 2011) and after a high-fat meal load (Koska *et al.* 2010), effects suggested to be GLP1 receptor and NO dependent (Nyström *et al.* 2004).

We previously reported that human coronary artery endothelial cells (HCAECs) express the GLP1 receptor and that exendin-4 is able to activate the receptor, leading to increased cell proliferation, involving the eNOS pathway (Erdogdu *et al.* 2010). GLP1 has also been described as an anti-apoptotic factor in different cells, including cardiomyocytes (Ravassa *et al.* 2011), neuronal cells (Li *et al.* 2009), and vein endothelial cells (Schisano *et al.* 2012, Shiraki *et al.* 2012). The aim of our study was to investigate the putative protective effect of exendin-4 and GLP1 against lipooapoptosis of HCAECs and attempt to address the pathways involved in imparting such an effect.

Materials and methods

Cell culture and incubation

Normal primary HCAECs isolated from normal human coronary arteries (passage 5–13) obtained from Clonetics (Lonza, Walkersville, MD, USA) were grown in EGM-2 MV medium supplemented with hydrocortisone, human epidermal growth factor, 5% FBS, vascular endothelial

growth factor, human fibroblast growth factor-B, R3-IGF1, ascorbic acid, and gentamicin/amphotericin B at 37 °C in a humidified atmosphere (5% CO₂, 95% air) as recommended by the supplier. Confluent cultures were detached by trypsin-2-[2-(Bis(carboxymethyl)amino)ethyl-(carboxymethyl)amino]acetic acid and seeded onto tissue culture dishes for evaluation of apoptosis and western blotting.

To examine the effect of exendin-4 on apoptosis, eNOS, and Akt phosphorylation, HCAECs were grown to 90% confluence, followed by incubation overnight in serum-deficient EGM medium containing 0.5% FBS and 2 mM L-glutamine. L-NG-nitroarginine methyl ester hydrochloride (L-NAME) (1 mM), LY294002 (1 µM), Rp-cAMP[S] (10 µM), p-38 MAPK inhibitor SB203580 (10 µM), JNK inhibitor SP600125 (5 µM), Akt inhibitor IV (0.5 µM), (6R)-5,6,7,8-tetrahydrobiopterin dihydrochloride (BH4) (10 µM), and (±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox) (1 mM) or vehicle were added 1 h before palmitate (125 µM, a concentration that has recently been demonstrated to induce apoptosis (Artwohl *et al.* 2008)), and exendin-4 stimulation and continuously present during the 24-h incubation. All materials, unless stated, were purchased from Sigma-Aldrich.

NO measurement

Direct measurements of NO release from HCAECs were performed using the cell-impermeable fluorescence indicator DAF-2 as described (Nakatsubo *et al.* 1998). Cells were incubated in 12-well plates in the presence or absence of palmitate with/without exendin-4 or vehicle in the serum-deficient medium for 24 h. The cells were subsequently washed twice in KRB containing in (mM) 135 NaCl, 3.6 KCl, 5 NaHCO₃, 0.5 NaH₂PO₄, 0.5 MgCl₂, 1.5 CaCl₂, and 10 HEPES, pH 7.4, followed by an incubation with 5 µM DAF-2 in 0.5 ml KRB for 2 h, at 37 °C, using the eNOS substrate L-arginine (100 µM) as positive control. At the end of the incubation, supernatants were transferred into black microplates and the fluorescence was measured with a fluorescence microplate reader Infinite M200 (Tecan Group Ltd., Männedorf, Germany) at excitation wavelength of 488 nm and emission 515 nm. Results were normalized to the protein concentrations determined using BCA kits after the cells in each well were lysed in a lysis buffer containing in (mM) 80 Na₂HPO₄, 20 NaH₂PO₄, 100 NaCl, and 1% Triton X-100, pH 7.5.

Western blot analysis

Western blotting was applied to quantify the total and phosphorylated eNOS (Ser¹¹⁷⁷), Akt 1/2/3 (Ser⁴⁷³), p38 MAPK (Thr¹⁸⁰/Tyr¹⁸²), or JNK (Thr¹⁸³/Tyr¹⁸⁵) proteins and performed as described previously (Zhang & Hintze 2006).

Measurement of intracellular reactive oxygen species

Intracellular reactive oxygen species (ROS) levels were measured using Image-iT LIVE Green Reactive Oxygen Species Detection Kit (Molecular Probes, Life Technologies Europe BV) as described previously (Suh *et al.* 2010). Briefly, the assay is based on 5-(and-6)-carboxy-2', 7'-dichlorodihydrofluorescein diacetate (carboxy-H2DCFDA), a fluorogenic marker that will be cleaved upon the presence of ROS. HCAECs were seeded into six-well plates. When reaching 80% confluence, cells were first kept overnight in serum-deficient EGM medium containing 0.5% FBS and 2 mM L-glutamine followed by a 24-h incubation in the presence or absence of palmitate or vehicle, with or without exendin-4. Cells were then washed with Hank's balanced salt solution (HBSS) before adding 50 μ M carboxy-H2DCFDA to each well. After 30 min of incubation at 37 °C, excess probe was removed by washing the cells again with HBSS. HCAECs were then lysed in PBS containing 1% Triton X-100. Carboxy-DCF fluorescence in cell lysates was detected at an excitation/emission wavelength of 495/529 nm using a microplate reader (Tecan Group Ltd.). The fluorescence intensity was normalized against the protein concentration of each individual well.

Determination of caspase-3 activity

Caspase-3 activity, as a measure of apoptosis, was determined using a fluorometric substrate, Z-DEVD-AMC (EnzChek Caspase-3 assay kit Molecular probes, Life Technologies Europe BV) as described previously (Lincz 1998). Twenty-four hours after stimulation with palmitate or vehicle w/wo exendin-4, cells were incubated in 50 μ l Z-DEVD-AMC substrate at room temperature for 30 min. Substrate cleavage was quantified fluorometrically at 342 nm excitation and 441 nm emission with a fluorescent plate reader (Tecan Group Ltd.).

DNA fragmentation

DNA fragmentation, another marker of apoptosis, in HCAECs was assayed by the cell death detection kit

ELISA plus (Roche Diagnostics Scandinavia AB), according to the manufacturer's instructions. The ELISA measures cytoplasmic DNA-histone complexes that increase after apoptosis-associated DNA fragmentation.

Figure 1

Exendin-4 blocks increased apoptosis induced by palmitate in HCAECs. Lipoapoptosis depends on the exposure time (A). Caspase-3 activation (B) and DNA fragmentation (C) measured in HCAECs exposed to palmitate (125 μ M) for 24 h, alone or in combination with different concentrations of exendin-4 (0.1–10 nM). Results are normalized by protein concentration in the incubation wells and expressed as mean percentage of control $\pm$ s.e.m. * P < 0.05 for a chance difference vs controls by ANOVA, * P < 0.05 vs palmitate. Results derived from seven (A and B) or six (C) independent experiments are shown.

Gene silencing

The cells were seeded into a 100 mm dish at a density of 2.5×10^5 cells per well and incubated for 24 h at 37 °C in complete medium. The cells were washed twice with culture medium without serum and supplement. Control siRNA/eNOS siRNA (10 nM) was mixed and incubated with SilenceMag according to the standard protocol (Oz Biosciences, Marseille, France). After incubation with a magnetic field for 15 min, the magnet was removed from the culture plate. Eight to twenty-four hours post-transfection, the media in the cell culture plate were replaced with complete medium containing 5% FBS and then further incubated for 24 h. The cells were harvested and centrifuged at 200 g for 2 min to remove the supernatant.

Isolation of total RNA and real-time PCR

Total RNA was extracted from HCAECs treated with eNOS siRNA and control siRNA, using Aurum Total RNA Mini kit Bio-Rad, according to the manufacturer's instructions. cDNA was prepared using Script cDNA Synthesis kit, Bio-Rad (Life Science Research). The PCR mixture contained, in a final volume of 20 µl, 4 µl cDNA, 10 µl KAPA SYBR FAST qPCR mastermix (Kapa Biosystems, Woburn, MA, USA), and corresponding primers. The gene expression level was normalized to the housekeeping gene, β -actin.

Statistical analyses

Each assay was repeated a minimum of three times. All data are presented as mean $\pm$ S.E.M. Statistical analyses of differences between groups were performed by one-way and two-way ANOVA followed by the *post hoc* tests Student–Newman–Keuls and Dunn–Bonferroni.

Results

Exendin-4 decreases HCAEC lipoapoptosis (caspase-3 activation and DNA fragmentation)

As shown in Fig. 1A, lipoapoptosis depends on its exposure time, cells incubated with 125 µM palmitate for 24 h in serum-deficient EGM medium (containing 0.5% FBS and 2 mM L-glutamine) exhibited the most significant increase in DNA fragmentation compared with other time points, as well as the control cells. All subsequent experiments were therefore conducted using 125 µM palmitate over 24 h. By contrast, palmitate-exposed

cells co-incubated with exendin-4 at various concentrations (0.1–10 nM) displayed a tendency to decrease caspase-3 activation compared with cells incubated with palmitate alone; however, the difference was not statistically significant (Fig. 1B). All experiments were conducted using 125 µM palmitate over 24 h. Figure 1C shows that DNA fragmentation was also increased in HCAECs incubated with 125 µM palmitate compared with control cells. This increase was blunted when cells were co-incubated with 10 nM exendin-4 (Fig. 1C).

Exendin-4 and GLP1 protect HCAECs from palmitate-induced apoptosis via a GLP1 receptor-dependent pathway

Many effects of GLP1 on the heart and the endothelium appear to be due to the formation of the DPP-4 metabolite GLP1 (9–36) (Ban *et al.* 2008, Nathanson *et al.* 2009). In order to examine whether the effects of exendin-4 were conveyed through a GLP1 receptor-dependent pathway, we introduced GLP1, its major metabolite GLP1 (9–36), and the GLP1 receptor antagonist exendin (9–39). Incubating the cells with palmitate and exendin-4 in the

Figure 2

The protective effect of exendin-4 and GLP1 against lipoapoptosis is mediated through the GLP1 receptor. DNA fragmentation measured in HCAECs stimulated w/wo palmitate (125 µM) for 24 h in the presence or absence of the indicated peptides (exendin-4, 10 nM; exendin (9–39), 10 nM; GLP1 (7–36), 100 nM, added every 12 h; GLP1 (9–36), 100 nM). Results are expressed as mean percentage of control $\pm$ S.E.M. * $P < 0.05$ vs control; # $P < 0.05$ vs palmitate. Results derived from 4 to 11 independent experiments are shown.

presence of GLP1 receptor antagonist exendin (9–39) led to loss of exendin-4 protection against lipoapoptosis (Fig. 2). Incubation of the cells with GLP1 also protected against palmitate-induced apoptosis but with a greater potency than that conferred by exendin-4. Interestingly, the GLP1 (9–36) metabolite was not able to protect the HCAECs against apoptosis (Fig. 2).

The anti-apoptotic action of exendin-4 is mediated by activation of PKA- and PI3K/Akt-dependent signaling pathways

Most of the activities initiated by binding of exendin-4 to its receptor on the surface of cells are mediated by an elevation in intracellular cAMP levels and PKA activity. We therefore examined whether blocking PKA activation countered the anti-apoptotic effect imposed by exendin-4. Cells were incubated with 125 μ M palmitate for 24 h in the presence or absence of 10 μ M Rp-cAMP[S], a specific inhibitor of PKA, and the extent of apoptosis was analyzed (Fig. 3A). Incubating the cells with Rp-cAMP[S] alone did not affect cellular apoptosis. However, when cells were incubated with palmitate and exendin-4 in the presence of Rp-cAMP[S], the anti-apoptotic action of exendin-4 was completely lost (Fig. 3A), indicating that the protective effect of exendin-4 against lipoapoptosis requires cAMP/PKA activation. In order to address whether cAMP could mimic the effect of exendin-4, we introduced a PKA agonist, Sp-cAMP[S]. Consistent with the above results, Sp-cAMP[S] blocked the increase in apoptosis induced by palmitate with an efficacy similar to that of exendin-4 (Fig. 3B). As we recently showed that exendin-4 activates eNOS through Akt phosphorylation in HCAECs (Erdogdu *et al.* 2010), we investigated whether palmitate exerts any effect on basal and exendin-4 stimulated Akt activation, a downstream kinase of the PI3K pathway, which was monitored by phosphorylation at Ser⁴⁷³. We observed that, while palmitate alone had no effects on Akt phosphorylation at Ser⁴⁷³, exendin-4 enhanced Akt phosphorylation at Ser⁴⁷³ (Fig. 4A). By contrast, incubating the cells with palmitate caused a slight, but statistically significant, increase in Akt protein expression. In addition, exposure to both palmitate and exendin-4 further augmented expression of Akt (data not shown). We next examined the role of PI3K and Akt in the prevention of apoptosis. Cells were incubated with 125 μ M palmitate in the presence or absence of LY294002 and Akt IV (which are selectively blocking PI3K and Akt respectively) and the extent of apoptosis was analyzed. LY294002 and Akt IV alone did not affect cellular apoptosis. However,

both LY294002 (Fig. 4B) and Akt IV (Fig. 4C) countered the protection against the DNA fragmentation induced by palmitate, indicating that this anti-apoptotic effect of exendin-4 involves PI3K/Akt signaling pathways.

Figure 3

PKA involvement in the protective effect of exendin-4 against lipoapoptosis. Cells were incubated for 24 h with palmitate (125 μ M) and/or the PKA antagonist Rp-cAMP[S] (A, 10 μ M) or the PKA agonist Sp-cAMP[S] (B, 10 μ M) in the presence or absence of exendin-4 (10 nM). Results are expressed as mean percentage of control $\pm$ s.e.m. * P < 0.05 vs control; # P < 0.05 vs palmitate. Results derived from five to six independent experiments (each in duplicate) are shown.

Palmitate enhances basal eNOS phosphorylation

To determine whether palmitate and exendin-4 (alone and in combination) influences eNOS activity, we examined activation, in a time course manner up to

24 h, of the enzyme by measuring its phosphorylation at Ser¹¹⁷⁷. As shown in Fig. 5A, enhanced phosphorylation of eNOS was observed already after 10 min and further on with palmitate. By contrast, exendin-4 enhanced eNOS activation after 30 min with a decreased activation after 24 h (Fig. 5A). Entirely consistent with the above findings, NO production by the HCAECs was significantly enhanced by palmitate (Fig. 5B). Furthermore, co-incubation of the cells with palmitate and exendin-4 further augmented NO production, approaching the effect produced by the eNOS substrate L-arginine (Fig. 5B).

Exendin-4 decreases palmitate-induced ROS production

As it is known that oxidative stress can elicit apoptosis, we decided to examine the influence of palmitate and exendin-4 on intracellular ROS production in the HCAECs. The cellular ability to produce ROS was measured by DCF fluorescence. This reaction is mainly started by hydrogen peroxide (Royall & Ischiropoulos 1993). Similarly, as in the case of apoptosis, palmitate was found to increase DCF fluorescence compared with control cells. However, this enhancement was decreased when cells were incubated with exendin-4 (Fig. 5C). We next incubated cells with the essential reduced cofactor BH4, a key factor in eNOS catalysis, i.e. the ratio between reduced and oxidized BH4 tightly control ROS formation. Restoration of BH4 also, to some extent, reduced ROS production (Fig. 5D) to the same level as observed by exendin-4 (Fig. 5C).

Protective effect of exendin-4 against lipoapoptosis is mediated by eNOS activation

We next investigated whether blocking eNOS activation affected the anti-apoptotic effect of exendin-4. Cells were incubated with palmitate for 24 h in the presence or absence of the eNOS inhibitor L-NAME (1 mM) and rates of

Figure 4

Involvement of PI3K and Akt in the anti-apoptotic effect of exendin-4. Cells were incubated with palmitate (125 μ M) for 24 h in the presence or absence of exendin-4 (10 nM) as indicated and phosphorylation of Akt (p-Akt) measured. The bar plot (A) shows the summarized data of the band intensities after being normalized to total Akt protein and α -tubulin. DNA fragmentation was measured in HCAECs w/o palmitate (0.125 mM) for 24 h, alone or in combination with exendin-4 (10 nM), in the absence or presence of the PI3K inhibitor LY294002 (B, 1 μ M) or the Akt IV inhibitor (C, 0.5 μ M). Results are expressed as mean percentage of control $\pm$ s.e.m. * P < 0.05 vs control; # P < 0.05 vs palmitate. Representative data from three to eight independent experiments are shown.

Figure 5

Involvement of NO in lipoapoptosis and protection by exendin-4. Cells were first incubated, in a time course manner, with palmitate (125 μM) in the presence or absence of exendin-4 (10 nM) as indicated (A). The bar plot (A) shows the summarized data of the band intensities of p-eNOS after being normalized to eNOS protein. 24-h incubation with exendin-4 significantly decreased eNOS activity (A), concomitant with an increase in NO production in cells treated with palmitate as shown in (B). The NO released into the buffer was measured and normalized to the protein content in each well. The effect of exendin-4 on palmitate-induced ROS production was also decreased as shown in (C), an effect that was mimicked

after incubation with the eNOS cofactor BH4 (D), suggesting that some of the effect might be due to an uncoupled eNOS. (E, F and G) The protective effect of exendin-4 against lipoapoptosis is eNOS dependent. DNA fragmentation was measured in HCAECs stimulated w/o palmitate (125 μM) for 24 h, alone or in combination with exendin-4 (10 nM), in the presence or absence of specific eNOS inhibitor L-NAME 1 mM (E), control siRNA (F), or eNOS siRNA transfection (G). Data are expressed as mean percentage of control ± S.E.M. **P* < 0.05 vs control; #*P* < 0.05 vs palmitate. Representative data from 3 to 12 independent experiments are shown.

apoptosis was analyzed (Fig. 5E). We have previously shown that L-NAME, at this concentration, effectively blocks eNOS activity and NO formation in these cells (Erdogdu *et al.* 2010). L-NAME alone did not affect cellular apoptosis and it did not modify palmitate-induced apoptosis. However, when cells were incubated with palmitate and exendin-4 in the presence of L-NAME, the incretin mimetic failed to prevent the increase in apoptosis induced by palmitate (Fig. 5E). To further corroborate the hypothesis that eNOS plays an important role in preventing lipoapoptosis, eNOS in HCAECs was silenced by siRNA. We transfected HCAECs with siRNA specific for eNOS or non-specific control (Lee *et al.* 2011). Knockdown of eNOS to 45% was observed by real-time PCR. As shown in Fig. 5F and G, the protective effect of exendin-4 on palmitate-induced apoptosis was completely

abolished by the eNOS siRNA. These findings altogether indicate that the protective effect of exendin-4 against lipoapoptosis in HCAECs is mediated by eNOS activation.

Exendin-4 inhibits palmitate-induced activation of p38 MAPK and JNK

p38 MAPK and JNK are involved in stress responses by regulating many cellular functions. As p38 MAPK and JNK have been demonstrated to be involved in the development of insulin resistance and cardiovascular diseases (Seeger *et al.* 2005), we examined the effect of palmitate on all three major MAPKs (p38 MAPK, JNK, and ERK1/2) because all are involved in the regulation of cell survival and apoptosis. p38 MAPK and JNK can be activated by phosphorylation at Thr¹⁸⁰/Tyr¹⁸² and Thr¹⁸³/Tyr¹⁸⁵

respectively, thereby regulating its catalytic activity. As shown in Fig. 6A and B, palmitate caused a significant increase in both p38 MAPK and JNK phosphorylation compared with control cells. This increment was significantly reduced by co-incubation with palmitate and exendin-4. However, incubating the cells with palmitate with/without exendin-4 did not significantly alter the phosphorylation of ERK1/2 (data not shown).

Figure 6

Exendin-4 inhibits palmitate-induced activation of p38 MAPK and JNK. Cells were incubated with palmitate (125 μ M) for 24 h in the presence or absence of exendin-4 (10 nM) as indicated. The bar plot shows the summarized data of the band intensities after being normalized against the total levels of p38 MAPK or JNK protein respectively and is expressed as mean percentage of control $\pm$ S.E.M. Results derived from four (A) or five (B) independent experiments are shown. * $P < 0.05$ vs control; # $P < 0.05$ vs palmitate.

The anti-apoptotic effect of exendin-4 is mediated by p38 MAPK- and JNK-dependent mechanisms

We next examined whether blocking p38 MAPK activation affected the protective effect of exendin-4 against lipoapoptosis. HCAECs were incubated with 125 μ M palmitate for 24 h in the presence or absence of 10 μ M SB203580, a specific inhibitor for p38 MAPK, and the extent of apoptosis was analyzed (Fig. 7A). As mentioned previously, palmitate induced a robust increase in apoptosis, which was attenuated by exendin-4. However, SB203580 alone significantly reduced cellular apoptosis. SB203580 at 10 μ M abolished some of the palmitate-induced apoptosis. By contrast, when cells were incubated with palmitate and exendin-4 in the presence of SB203580, the anti-apoptotic effect of exendin-4 was blocked by co-incubation with SB203580. As JNK has also been shown to mediate cellular apoptosis, we examined whether blocking JNK activation by SP600125, a specific inhibitor for JNK, also abolished the protective effect of exendin-4 on palmitate-induced apoptosis. Our results indicate that, in the presence of SP600125, exendin-4 failed to prevent the increase in apoptosis induced by palmitate (Fig. 7B). We next incubated cells with both SB203580 (10 μ M) and SP600125 (5 μ M), which nearly completely abolished the induced apoptosis (Fig. 7C). Finally, we used an antioxidant (Trolox) that completely inhibited ROS production (Fig. 7D), with no such effect on apoptosis (Fig. 7E). However, our results indicate that, in the presence of SB203580 and SP600125, exendin-4 failed to prevent the increase in apoptosis induced by palmitate (Fig. 7C). Taken together, these data suggest that the anti-apoptotic effect of exendin-4 is dependent on p38 MAPK and JNK activities, however not necessarily due to oxidative stress.

Discussion

The main findings of this study are as follows: long-term exposure of HCAECs to palmitate induces apoptosis, eNOS activity, ROS release, and production of NO. Exendin-4 and GLP1, but not the degradation metabolite GLP1 (9–36), confer protection against this lipoapoptosis. The anti-apoptotic effect of exendin-4 is mediated through the GLP1 receptor and involves PKA-, PI3K-, eNOS-, p38 MAPK-, and JNK-dependent pathways.

Elevated levels of circulating FFAs frequently seen in patients with type 2 diabetes (Chai & Liu 2007) are suggested to decrease NO bioactivity, either by increased destruction or an impaired eNOS activity. As endothelial dysfunction is tightly coupled to a decrease in NO

Figure 7

Involvement of p38 MAPK and JNK in the anti-apoptotic effect of exendin-4. DNA fragmentation measured in HCAECs stimulated w/o palmitate (125 μM) for 24 h, alone or in combination with exendin-4 (10 nM), in the absence or presence of the p38 MAPK inhibitor SB203580 (10 μM) (A) or the JNK inhibitor SP600125 (5 μM) (B), or in combination (C). The antioxidant

Trolox (1 mM) completely abolished ROS production (D), without any effect on apoptosis (E). Results derived from four to six independent experiments (in duplicates) are shown and expressed as mean percentage of controls $\pm$ s.e.m. * P <0.05 vs control; # P <0.05 vs palmitate.

bioavailability (Harrison 1997), we would have expected palmitate to downregulate eNOS activity (Kim *et al.* 2005, Wang *et al.* 2006). Paradoxically, eNOS phosphorylation was robustly increased in HCAECs exposed to palmitate. Accordingly, NO production was also increased by palmitate and so was the release of ROS. Notably, a number of studies have demonstrated that NO release is increased under diabetic conditions (Xu & Zou 2009). It was recently shown that palmitate stimulates ROS production in aortic endothelial cells through PKC-dependent activation (Inoguchi *et al.* 2000). Usually, ROS are scavenged through multiple defense mechanisms, and excessive levels of ROS can impede these systems and promote breakdown NO, thereby exacerbating the oxidative stress (Tefamariam & Cohen 1992, Cosentino *et al.* 1997). In hyperlipidemic conditions, any of the three NOS enzymes might yield superoxide anion (usually referred to uncoupling of eNOS), known to react rapidly with NO,

leading to production of the highly pro-oxidant peroxynitrite (Zou *et al.* 2004). Peroxynitrite is known to be toxic and triggers many different cellular responses including apoptosis of the cells (Laursen *et al.* 2001, Zou *et al.* 2004). It was recently demonstrated that treatment of human aortic endothelial cells with high concentrations of glucose increases the eNOS expression (due to uncoupling of the enzyme) and subsequently causes oxidative stress (Cosentino *et al.* 1997). Moreover, short-term exposure to a triglyceride-rich fat emulsion, mimicking the elevated FFAs in our current *in vitro* work, impairs endothelium-dependent vasorelaxation, with a concomitant increase in eNOS activity in rat conduit artery rings *ex vivo* (Nathanson *et al.* 2009). Our results confirm the above and previous suggestions, i.e. palmitate induces an imbalance between NO and ROS release, and that the enhancement of eNOS activity might be due to uncoupling of this enzyme. One causative factor for eNOS

uncoupling is a deficient amount of the essential cofactor of eNOS, i.e. BH4 (Verhaar *et al.* 2004). Supporting that notion, eNOS activity was substantially decreased by exendin-4 treatment in our study, with a concomitant reduction in ROS levels and mimicked by BH4 restoration. By contrast, prolonged exposure of the HCAECs to exendin-4 further augmented NO production, a crucial factor in regulation and coordination of endothelial function and apoptosis (Calles-Escandon & Cipolla 2001).

GLP1 and its derivatives have previously been assigned an anti-apoptotic role in murine HL-1 cardiomyocytes (Ravassa *et al.* 2011), pancreatic β -cells (Cunha *et al.* 2009, Kim *et al.* 2010), and neuronal cells (Li *et al.* 2009). Very recently, it was also shown that exendin-4 protects pancreatic β -cells against lipoapoptosis by interfering with the JNK signaling pathway. Recent studies have revealed differential effects of exendin-4 and GLP1 on vascular functions (Ban *et al.* 2008, Nathanson *et al.* 2009), suggesting the existence of a signaling pathway independent of the classical GLP1 receptor. To this end, we attempted to address whether the anti-apoptotic effect of exendin-4 was mediated by the GLP1 receptor using the GLP1 receptor antagonist exendin (9–39). The anti-apoptotic effect induced by exendin-4 was indeed abolished by the GLP1 receptor antagonist, thus suggesting GLP1 receptor-dependent action of the peptide. Interestingly, the major metabolite GLP1 (9–36), which possesses no affinity to the classical GLP1 receptor (Knudsen & Pridal 1996, Ban *et al.* 2008), failed to reduce palmitate-induced apoptosis in HCAECs, adding further credence in favor of the view that the protective effect of exendin-4 is mediated through the known GLP1 receptor in these cells. It was previously reported that most effects of GLP1 are mediated through activation of cAMP/PKA signaling (Brubaker & Drucker 2004). Using a PKA inhibitor and activator, we show that PKA activation is both sufficient and required for exendin-4 to confer its protective actions. Thus, stimulation of the PKA pathway by exendin-4 may convey the protective effect of the peptide against lipoapoptosis in HCAECs.

Further downstream the PI3K/Akt pathway is known to be involved in protection from apoptosis in different cell types (Piro *et al.* 2008). Akt plays a crucial role in insulin signaling and its suppression can lead to both insulin resistance and apoptosis (Piro *et al.* 2008). By contrast, palmitate did not influence Akt phosphorylation in the current work. The addition of exendin-4 significantly enhanced phosphorylation of Akt and decreased the amount of apoptotic cells. Furthermore, the anti-apoptotic effect of exendin-4 was prevented when

endothelial cells were cultured with LY294002 and Akt inhibitor IV respectively, suggesting that exendin-4 requires activation of the PI3K/Akt pathway to protect HCAECs from lipoapoptosis. Consistent with our findings, previous findings in other cell types also indicate that the anti-apoptotic actions of GLP1 are mediated through a PI3K-dependent signaling pathway (Kimura *et al.* 2009).

eNOS plays a pivotal role in endothelial cell proliferation and survival (Calles-Escandon & Cipolla 2001). This enzyme is activated by exendin-4 through phosphorylation, leading to enhanced NO production and this effect is dependent on the activation of the PI3K/Akt pathway (Erdogdu *et al.* 2010). In addition, eNOS can also be activated by PKA or Akt, resulting in increased NO production (Schulz *et al.* 2005). Previous work has indicated that the activity of eNOS is diminished in endothelial apoptosis induced by multiple proapoptotic factors (Dimmeler & Zeiher 1999). By contrast, we demonstrated in this study that paradoxically increased eNOS activity is associated with enhancement of lipoapoptosis and that co-incubation with exendin-4 attenuated this proapoptotic effect of palmitate. The anti-apoptotic effect of exendin-4 was abolished when HCAECs were cultured with L-NAME. In addition, genetic silencing of transfection of eNOS abrogated the protective effect of exendin-4 against lipoapoptosis, demonstrating that the anti-apoptotic action of exendin-4 is also mediated by an eNOS-dependent pathway in the HCAECs.

Recently, it was reported that high levels of ROS not only damage cells by oxidizing DNA and protein but also indirectly damage cells by activating a variety of stress-sensitive intracellular signaling pathways, such as p38 MAPK and JNK (Klaunig *et al.* 2010). In the current study, palmitate increased the phosphorylation of both p38 MAPK and JNK and apoptosis in HCAECs. Furthermore, co-incubation of the cells with exendin-4 significantly decreased the phosphorylation levels of these kinases, with a concomitant reduction in endothelial cell apoptosis. That inhibition of p38 MAPK and JNK with specific inhibitors completely abolished the proapoptotic effects of palmitate, suggesting that palmitate induces apoptosis in HCAECs through p38 MAPK- and JNK-dependent pathways. This finding is consistent with a recent report demonstrating that palmitate also triggers cytokine secretion via both p38 MAPK and JNK pathways in differentiated THP-1 cells, a model of human macrophages (Haversen *et al.* 2009). Recently, it was also shown that exendin-4 protects β -TC6 insulinoma cell line against lipoapoptosis by interfering with the JNK signaling pathway (Xiang *et al.* 2012). It has also been reported that p38 MAPK activation alone

accounts for palmitate-induced apoptosis in HCAECs (Chai & Liu 2007). Interestingly, the anti-apoptotic effect of exendin-4 was also abolished by specific inhibitors of p38 MAPK and JNK, indicating that activation of these kinases not only contributes to increased lipoapoptosis but also that their suppression plays an important role in HCAEC survival caused by exendin-4. In the current study, the antioxidant Trolox inhibited palmitate-induced ROS production, similar to the inhibitors of p38 MAPK and JNK kinases. Surprisingly, Trolox did not evoke any anti-apoptotic actions. Reason for that is not clear; however, we cannot exclude that other ROS are behind the involvement of p38 MAPK and JNK kinases upon apoptosis. Recently, it was demonstrated that exendin-4 inhibits cytotoxic induced β -cell death via a decreased phosphorylation of both p38 MAPK and JNK, through PKA signaling pathways (Kawasaki *et al.* 2010), which is in line with our observation.

In conclusion, we demonstrate that palmitate activates eNOS to stimulate NO release and that p38 MAPK and JNK signaling pathways are involved in palmitate-induced ROS production and lipoapoptosis. Exendin-4 and GLP1 protect HCAECs against lipoapoptosis, which is mediated through the GLP1 receptor involving PKA-, eNOS-, p38 MAPK-, and JNK-dependent pathways. These effects, demonstrated *in vitro*, might serve to limit the adverse consequences of the macrovascular complications of type 2 diabetes as dysfunction of endothelial cells is believed to contribute to premature development of atherosclerosis.

Declaration of interest

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Author contribution statement

All authors contributed to the study conception and design. Ö E, L E, H X, and Q Z conducted the study. All authors analyzed the data. Ö E, Å S, and T N wrote the first draft of the paper. L E conducted the additional experiments due to the revision. All authors commented and took part of the revision of the paper.

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