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Exendin-4 stimulates proliferation of human coronary artery endothelial cells through eNOS-, PKA- and PI3K/Akt-dependent pathways and requires GLP-1 receptor

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ABSTRACT

Endothelial cells have a robust capacity to proliferate and participate in angiogenesis, which underlies the maintenance of intimal layer integrity. We previously showed the presence of the GLP-1 receptor in human coronary artery endothelial cells (HCAECs) and the ameliorative actions of GLP-1 on endothelial dysfunction in type 2 diabetic patients. Here, we have studied the effect of exendin-4 on cell proliferation and its underlying mechanisms in HCAECs.

Incubation of HCAECs with exendin-4 resulted in a dose-dependent increase in DNA synthesis and an increased cell number, associated with an enhanced eNOS and Akt activation, which were inhibited by PKA, PI3K, Akt or eNOS inhibitors and abolished by a GLP-1 receptor antagonist. Similar effects were obtained by applying GLP-1 (7–36) or GLP-1 (9–36). Co-incubation of exendin-4 and GLP-1 did not show additive effects. Our results suggest that exendin-4 stimulates proliferation of HCAECs through PKA-PI3K/Akt-eNOS activation pathways via a GLP-1 receptor-dependent mechanism.

1. Introduction

The endothelium represents a single layer of cells that line the vessels in the body, protecting the vessels from activation of clotting and proinflammatory factors (Biegelsen and Loscalzo, 1999; Calles-Escandon and Cipolla, 2001) and participating in the regulation of blood flow and blood pressure. The disruption of normal vascular endothelial function mediates abnormal vasomotion, adhesion of monocytes and platelets and migration and proliferation of vascular smooth muscle cells (Calles-Escandon and Cipolla, 2001), resulting in enhanced coagulation, cell adhesion, inflammation and trans-endothelial transport of atherogenic lipoproteins, and inappropriate vasoconstriction or vasodilation (Rask-Madsen and King, 2007).

Endothelial cells have a robust capacity to grow and migrate and participate in angiogenesis, which underlies both neovascularization and the maintenance of intimal layer integrity (Kawasaki et al., 2003). Endothelial growth and apoptosis are involved in endothelial repair and function. Disruption of the intimal layer subjects the arterial wall at greater risk for vascular disease (Ross, 1993), the most common etiology for morbidity and mortality in diabetic patients (Marshall and Flyvbjerg, 2006). There is a mechanistic link between enhanced endothelial cell turn-over and the susceptibility to atherosclerotic plaque development (Calles-Escandon and Cipolla, 2001; Caplan and Schwartz, 1973). The loss of endothelial integrity leads to vascular complications. Endothelial cell injury and proliferative dysfunction are considered to be the initial events in the development of atherosclerosis (Berk et al., 2001). Proliferation of endothelial cells adjacent to the site of injury is an important mechanism of endothelial repair. In addition, endothelial cell proliferation plays an important role in angiogenesis (Nishi et al., 2008), a physiological or pathological neovascularization process under certain conditions, such as tissue ischemia.

Glucagon-like peptide-1 (GLP-1) is a brain-gut insulinotropic peptide that plays an important role in the regulation of glucose homeostasis and thus has been applied in clinical treatment of diabetes (Wajchenberg, 2007; Drucker and Nauck, 2006). Native GLP-1 has a short half-life of a few minutes, being rapidly degraded by dipeptidyl peptidase-IV (DPPIV), to generate an NH_2-terminally truncated metabolite *i.e.*, GLP-1 (9–36), in addition to renal excretion. Exendin-4 is a stable GLP-1 analogue that is resistant to DPPIV (Drucker and Nauck, 2006) and is approved in both Europe and the U.S. for clinical treatment of type 2 diabetes. The biological actions of GLP-1 and exendin-4 are believed to be mediated through the GLP-1 receptor. We previously showed that the endothelial cells express the GLP-1 receptor and that acute administration of GLP-1 improves endothelial dysfunction in type 2 diabetes patients with

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coronary heart disease (Nystrom et al., 2004), suggesting an important role of GLP-1 in endothelial function. In stark contrast to its effects on glycemic end points, the role of GLP-1 and its analogues on endothelial cell growth and function remains elusive. In the present study, we have investigated the putative mitogenic effect of exendin-4, GLP-1 (7–36) and GLP-1 (9–36) on proliferation of human coronary artery endothelial cells (HCAEC), and attempted to address the signal transduction pathways involved in conveying such an effect.

2. Materials and methods

2.1. Materials

Antibodies against phospho-eNOS (Ser 1177), eNOS, phospho-Akt 1/2/3 (Ser 473)-R, Akt 1/2/3 and horseradish peroxidase-labeled goat anti-rabbit IgG, horseradish peroxidase-labeled goat anti-mouse IgG and the enhanced chemiluminescence ECL kit were from Santa Cruz Biotechnology (Santa Cruz, CA). Protease inhibitor cocktail was from Roche Diagnostics (Mannheim, Germany). Exendin-4, monoclonal anti-beta actin antibody, Rp-adenosine 3′,5′-cyclic monophosphorothioate triethylammonium salt hydrate (Rp-cAMP[S]) and N_ω-nitro-L-arginine methyl ester hydrochloride (L-NAME) were from Sigma–Aldrich (St Louis, MO). GLP-1 (7–36), GLP-1 (9–36) and exendin (9–39) were from PolyPeptide Laboratories (Torrance, CA). LY294002 and Akt inhibitor IV were from Calbiochem (La Jolla, CA). Re-blot plus strong solution was from Chemicon (Temecula, CA). Culture medium EGM-2 MV and cell culture supplements, Hepes buffer saline solution, and trypsin neutralizing solution (TNS) were purchased from Clonetics (Lonza, Walkersville, MD). *Methyl*-[^{3}H]thymidine (5 Ci/mmol) was from Amersham Biosciences (Piscataway, NJ). Bicinchoninic acid (BCA) kit was from Pierce Chemical Co. (Rockford, IL). Pathscan phospho-Akt1 sandwich ELISA kit, U0126 (MEK1/2 inhibitor), anti-phospho-MAP kinase 1/2 and anti-MAP kinase 1/2 were from Cell Signaling Technology (Danvers, MA).

2.2. Cell culture and incubation

Normal primary human coronary artery endothelial cells (HCAECs), isolated from normal human coronary arteries (passage 5–13) (Seli et al., 2006; Kondapalli et al., 2004; Li et al., 1998) obtained from Clonetics (Lonza, Walkersville, MD), were grown in EGM-2 MV medium supplemented with hydrocortisone, human epidermal growth factor (hEGF), 5% fetal bovine serum (FBS), vascular endothelial growth factor (VEGF), human fibroblast growth factor (hFGF)-B, R^3-insulin-like growth factor (IGF)-1, ascorbic acid and gentamicin/amphotericin-B at 37 °C in a humidified (5% CO_2, 95% air) atmosphere as recommended by the supplier. Confluent cultures were detached by trypsin-2-[2-(Bis(carboxymethyl)amino)ethyl-(carboxymethyl)amino]acetic acid (EDTA) and seeded onto tissue culture dishes for evaluation of [^{3}H]thymidine incorporation rates, cell counting and Western blotting.

To examine the effects of exendin-4, GLP-1 (7–36) and GLP-1 (9–36) on cell viability, DNA synthesis, eNOS, MAP kinase and Akt phosphorylation, HCAECs were grown to 90% confluence, followed by an incubation overnight in serum-deficient EGM medium containing 0.5% FBS and 2 mM L-glutamine. L-NAME (1 mM), LY294002 (2 μM), Rp-cAMP[S] (10 μM) or vehicle were added 30 min, and Akt inhibitor IV or U0126 were added 1 h, prior to exendin-4, GLP-1 (7–36) and GLP-1 (9–36) stimulation and continuously present as the incubation was continued for 48 h.

2.3. NO measurement

Direct measurement of nitric oxide (NO) release from HCAECs was 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 exendin-4 (10 nM) or the inhibitors (1 mM L-NAME, 2 μM LY294002 or vehicle) in the serum-deficient medium for 48 h. The cells were subsequently washed twice in Krebs-Ringer bicarbonate Hepes buffer (KRBH) buffer containing (in mM) 135 NaCl, 3.6 KCl, 5 $NaHCO_3$, 0.5 NaH_2PO_4, 0.5 $MgCl_2$, 1.5 $CaCl_2$ and 10 Hepes, pH 7.4, followed by an incubation with 5 μM DAF-2 in 0.5 ml KRBH buffer for 2 h, at 37 °C, using the eNOS substrate L-arginine (100 μM) as the positive control. Same concentrations of exendin-4 or the inhibitors were present in the corresponding wells during the incubation. At the end of the incubation, the supernatants were transferred into black microplates and the fluorescence was measured with a fluorescence microplate reader (Infinite M200, TECAN) 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 the lyses buffer containing (in mM) 80 Na_2HPO_4, 20 NaH_2PO_4, 100 NaCl, and 1% Triton X-100, pH 7.5.

2.4. Western blot analysis

Western blotting was applied to quantify the total and phosphorylated eNOS (Ser 1177) or Akt 1/2/3 (Ser 473) proteins and performed as previously described (Zhang et al., 2006). Briefly, HCAECs were grown and incubated as described above in 100-mm Petri dishes. Cells were washed twice with PBS and lysed on ice in ice-cold lysis buffer containing 1 mM sodium fluoride, 1 mM sodium orthovanadate, 1× protease inhibitor cocktail and 2% Triton X-100 in PBS, pH 7.5, for 30 min. The cell lysates were centrifuged (5000 rpm, 5 min, 4 °C) and the supernatant was collected. Equal amounts of protein (20–30 μg) were subjected to SDS–PAGE under reducing conditions. The separated proteins were electrotransferred onto nitrocellulose membranes. The membranes were blocked in TBS-T (20 mM Tris–base, 137 mM NaCl, pH 7.6, with 0.05% Tween 20) with 5% non-fat dry milk, followed by an overnight incubation with anti-phospho-eNOS (Ser 1177) or phospho-Akt antibodies (1:500) in TBS-T/1% BSA at 4 °C. The membranes were extensively washed and subsequently incubated with peroxidase-conjugated goat anti-rabbit IgG (1:10,000) in TBS-T with 1% BSA for 1 h at room temperature. The membranes were extensively washed and the immunostained proteins were visualized by ECL. The blots were stripped in re-blot plus strong solution and probed with either anti-phospho-Akt 1/2/3 (Ser 473) (1:500), anti-eNOS (1:500) or anti-β-actin. The intensities of the bands were quantified by densitometry (Gel Doc™, Bio-Rad laboratories, with software Quantity One).

2.5. Measurement of Akt activity

Phospho-Akt was measured using Pathscan phospho-Akt1 sandwich ELISA kit, according to the manufacturer's instructions. The phospho-Akt specific ELISA detects Akt phosphorylated at serine 473. Samples were prepared from cells after a 48-h incubation in the presence or absence of GLP-1 (7–36) and 100 μl of samples containing equal amount of protein were applied to each well.

2.6. [^{3}H]thymidine incorporation

Rates of [^{3}H]thymidine incorporation into DNA were analyzed as previously described (Edwards et al., 2008). In brief, cells were grown in 60-mm Petri dishes and cultured until 90% confluence.