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Glucagon-like peptide-1 inhibits vascular smooth muscle cell dedifferentiation through mitochondrial dynamics regulation

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ABSTRACT

Glucagon-like peptide-1 (GLP-1) is a neuroendocrine hormone produced by gastrointestinal tract in response to food ingestion. GLP-1 plays a very important role in the glucose homeostasis by stimulating glucose-dependent insulin secretion, inhibiting glucagon secretion, inhibiting gastric emptying, reducing appetite and food intake. Because of these actions, the GLP-1 peptide-mimetic exenatide is one of the most promising new medicines for the treatment of type 2 diabetes. *In vivo* treatments with GLP-1 or exenatide prevent neo-intima layer formation in response to endothelial damage and atherosclerotic lesion formation in aortic tissue. Whether GLP-1 modulates vascular smooth muscle cell (VSMC) migration and proliferation by controlling mitochondrial dynamics is unknown. In this report, we showed that GLP-1 increased mitochondrial fusion and activity in a PKA-dependent manner in the VSMC cell line A7r5. GLP-1 induced a Ser-637 phosphorylation in the mitochondrial fission protein Drp1, and decreased Drp1 mitochondrial localization. GLP-1 inhibited PDGF-BB-induced VSMC migration and proliferation, actions inhibited by overexpressing wild type Drp1 and mimicked by the Drp1 inhibitor Mdivi-1 and by overexpressing dominant negative Drp1. These results show that GLP-1 stimulates mitochondrial fusion, increases mitochondrial activity and decreases PDGF-BB-induced VSMC dedifferentiation by a PKA/Drp1 signaling pathway. Our data suggest that GLP-1 inhibits vascular remodeling through a mitochondrial dynamics-dependent mechanism.

1. Introduction

The incretin glucagon-like peptide-1 (GLP-1) is a hormone released from the gut in response to food intake and was initially described as an agent favoring post-prandial insulin release [1,2]. Due to its insulinotropic action, GLP-1 mimetics were developed and approved as a therapy for controlling hyperglycemia in type 2 diabetes [3]. The main physiological effects of GLP-1 or GLP-1 mimetics are related to its incretin property [4]. However, extrapancreatic effects of incretin has been also described, particularly in the central nervous system, heart, adipose tissue, skeletal muscle, endothelial cells and vascular smooth muscle cells (VSMCs) [5,6].

We hypothesized that mitochondrial dynamics and metabolism are important for VSMC differentiation–dedifferentiation [7]. In fact, changes in mitochondrial dynamics have been implicated in VSMC proliferation and contractile protein expression [7], suggesting that mitochondrial morphology is relevant for VSMC phenotypic modulation. Modulation of VSMC phenotype is involved in the development and progression of atherosclerosis, hypertension and neointimal formation [8,9].

Mitochondrial dynamics involves the transition between elongated interconnected mitochondrial networks and a

Abbreviations: $\Delta\Psi_m$, mitochondrial membrane potential; Drp1, dynamin related protein 1; Fis1, fission protein 1; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GLP-1, glucagon-like peptide-1; Mfn1/2, mitofusin-1/2; MTO, MitoTracker Orange; OCR, oxygen consumption rate; Opa1, optic atrophy 1 protein; PDGF-BB, platelet-derived growth factor-BB; PKA, protein kinase A; ROS, reactive oxygen species; VSMC, vascular smooth muscle cell.

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fragmented disconnected arrangement by events of mitochondrial fusion and fission, respectively [10]. Mitochondrial fission is regulated by the fission protein 1 (Fis1) and the dynamin-related protein 1 (Drp1). Fis1 is localized in the mitochondrial outer membrane and recruits cytoplasmic Drp1 under fission-promoting stimuli, triggering the constriction of mitochondrial membranes [10,11]. In contrast, fusion is controlled by the large GTPases mitofusins 1/2 (Mfn1/2) and optic atrophy 1 protein (Opa1), promoting fusion of the outer and inner membranes, respectively [10,11]. Recently, we showed that GLP-1 enhances the functional coupling between endoplasmic reticulum (ER) and mitochondria in VSMC [12]. However, whether GLP-1 controls mitochondrial dynamics and activity and therefore regulates VSMC phenotypic modulation remains unknown.

To address this, we assessed the effects of GLP-1 on mitochondrial dynamics and activity, and its effects on VSMC phenotype. Our data showed that GLP-1 stimulates mitochondrial fusion and activity through a protein kinase A (PKA)-dependent Drp1 inactivation and through the increase of Mfn2 protein levels. PKA inhibition completely blocked GLP-1-induced mitochondrial fusion and activity. Importantly, GLP-1 abolished platelet-derived growth factor (PDGF)-BB-induced migration and proliferation through a PKA/Drp1 signaling pathway. Altogether, these results suggest that mitochondrial morphology and function regulate migratory effects of GLP-1 on vascular smooth muscle A7r5 cell line.

2. Materials and methods

2.1. Cell culture

The A7r5 cell line, originally derived from embryonic rat aorta, was purchased from the American Type Culture Collection (ATCC) and cultured as described [12]. Prior to stimulation, 80–90% confluent VSMCs were serum-starved overnight and then stimulated with 100 nM GLP-1 (7-36) amide (cat # 46113, American Peptide Company, Sunnyvale, CA, USA).

2.2. Mitochondria imaging

After stimulation with GLP-1, cells were incubated with MitoTracker Orange (MTO, 400 nM for 30 min [cat # M7510, Molecular Probes-Invitrogen, Eugene, OR, USA]), washed with PBS, fixed with paraformaldehyde (4% [w/v] in PBS for 20 min [cat # 15700, Electron Microscopy Sciences, Hatfield, PA, USA]) and blocked with bovine serum albumin (BSA, 1% [w/v] in PBS for 1 h [cat # 05470, Sigma–Aldrich Corp., St. Louis, MO, USA]). Confocal images were captured with a Zeiss LSM-5, Pascal 5 Axiovert 200, equipped with a Plan-Apochromat 63×/1.4 Oil DIC objective and with LSM 5 3.2 software for image capture/analysis as previously described [12]. Providing a pixel size of 0.0049 μm^2 and according to Nyqvist criterion, the resulting optical resolution (*xy*) is 143 nm. With this resolution, we can track changes in mitochondrial size that ranged from 0.039 to 0.873 μm^2, as described for other cells in the cardiovascular system [13]. Images were deconvolved with Image J (NIH) and then, Z-stacks of thresholded images were volume-reconstituted using the VolumeJ plug-in. The number and individual volume of each object (mitochondria) were quantified using the ImageJ-3D Object counter plug-in. Each experiment was done at least four times and each time 16–25 cells per condition were quantified. A decrease in mitochondrial volume or area and an increase in the number of mitochondria per cell were considered as fission criteria [14,15].

2.3. Mitochondrial activity

Mitochondrial membrane potential ($\Delta\Psi_m$), reactive oxygen species (ROS), ATP content and oxygen consumption were determined as described [12].

2.4. Drp1 and mitochondria colocalization

A7r5 cells cultured on coverslips were stimulated with GLP-1 for 3 h. During the last 30 min of treatment 400 nM MTO was added. Cells were washed with PBS and fixed with PBS containing 4% paraformaldehyde and incubated for 10 min in ice-cold 0.3% Triton X-100 (cat # X100, Sigma–Aldrich Corp., St. Louis, MO, USA) for permeabilization. Nonspecific sites were blocked with 1% BSA in PBS for 1 h and then the cells were incubated with anti Drp1 (1:500) antibody (cat # 611112, BD Transduction Laboratories, San Jose, CA, USA). Secondary antibody was anti-mouse Alexa488 (cat # A-11034, Molecular Probes-Invitrogen, Eugene, OR, USA). For the colocalization analysis only one focal plane was analyzed with a Zeiss LSM-5 Pascal 5 Axiovert 200 microscope. Images obtained were deconvolved and background was subtracted using the ImageJ software. Colocalization between the Drp1 and mitochondria was quantified using the Manders' algorithm, as previously described [15,16].

2.5. Western blot analysis

Equal amounts of protein from cells were separated by SDS–PAGE (10% polyacrylamide gels) and electrotransferred to nitrocellulose. Membranes were blocked with 5% defatted milk in Tris-buffered saline, pH 7.6, containing 0.1% (v/v) Tween 20 (TBST). Membranes were incubated with primary antibodies at 4 °C (mtHsp70 1:1000 [cat # MA3-028, ABR-Affinity Bioreagents, Glosen, CO, USA], total Drp1 1:1000 [cat # 611112, BD Transduction Laboratories, San Jose, CA, USA], p-Drp1 1:500 [cat # 4867, Cell Signalling, Danvers, MA, USA], Mfn2 1:1000 [cat # ab50838, Abcam, Cambridge, MA, USA]; Fis1 1:1000 [cat # ALX 210 907, Alexis Biochemicals, San Diego, CA, USA], Opa1 1:1000 [cat # ab55772, Abcam, Cambridge, MA, USA]; β-tubulin 1:5000 [cat # T0198, Sigma–Aldrich Corp., St. Louis, MO, USA]; β-actin 1:5000 [cat # A3853, Sigma–Aldrich Corp., St. Louis, MO, USA]) and re-blotted with horseradish peroxidase-linked secondary antibody (1:5000 in 1% [w/v] defatted milk in TBST [cat # AP307P & AP308P, Calbiochem, La Jolla, CA, USA]). The bands were detected using ECL (cat # NEL103001EA, Perkin Elmer, Waltham, MA, USA) with exposure to Kodak film and quantified by scanning densitometry. Protein content was normalized by β-tubulin or β-actin.

2.6. Immunoprecipitation of Drp1

For immunoprecipitation of Drp1, 0.4 mg of total protein lysate of each treatment condition was mixed with 30 μL of protein A/G PLUS-agarose (cat # sc-2003, Santa Cruz Biotechnology, Dallas, TX, USA) and 30 μL anti total Drp1 antibody and incubated for overnight at 4 °C. After centrifugation at 9000 × *g* for 5 min at 4 °C, the elution of Drp1 was performed by adding 15 μL of 4x SDS-sample buffer and boiling. Equal amounts of supernatants were submitted to western blotting analysis and revealed using anti p-Drp1 antibody.

2.7. Adenoviruses

A7r5 cells were transduced with adenoviruses overexpressing β-galactosidase (LacZ), wild type Drp1 (wtDrp1), dominant negative Drp1 (DN-Drp1) and wild type Mfn2 (Mfn2) for 24 h using MOI 300.