



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.

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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 Nyquist 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 \times 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.

2.8. Migration assay

Near confluent A7r5 cells seeded in 60 mm culture plates were serum deprived for 24 h. Culture media was replaced with DMEM containing bromodeoxyuridine (100 μ M [cat # B9285, Sigma–Aldrich Corp., St. Louis, MO, USA]) then treated with GLP-1 (100 nM), forskolin (100 μ M [cat # F6886, Sigma–Aldrich Corp., St. Louis, MO, USA]) or Mdivi-1 (10 μ M [cat # M0199, Sigma–Aldrich Corp., St. Louis, MO, USA]) for 3 h and then stimulated with PDGF-BB (10 nM [cat # 521225, Merck Millipore Corporation, Darmstadt, Germany]) for another 24 h. Just after adding PDGF-BB, a sterile 200 μ L pipet tip was used to make a straight scratch in the cells in each plate. Inhibitors (H-89 100 nM [cat. 371963, Calbiochem, La Jolla, CA, USA]; Exendin-9 [Ex-9] 100 nM [cat # 46310B, American Peptide Company, Sunnyvale, CA, USA]) were added 30 min before GLP-1 pretreatment. Under a 10 \times lens, images were taken of 9 fields per well of the linear wound. PDGF-BB-stimulated migration was expressed as a percentage of migration respect to the initial distance at time 0 h. Alternatively, cell migration was assessed using 24-well plates with Transwell inserts (8.0 μ m pore; Costar [cat # 3422, Corning Incorporated, NY, USA]), as described previously [17].

2.9. Proliferation assay

VSMC proliferation was determined by [³H]-thymidine incorporation. Cells were pulse-labeled for 4 h in culture medium containing [³H]-thymidine (1 μ Ci/mL, 2 Ci/mmol [cat # NET027A001MC, Perkin Elmer, Waltham, MA, USA]) as described [18]. Each experiment was performed in triplicate in 6-well tissue culture plates.

2.10. Statistical analysis

Data are shown as mean \pm SEM of the number of independent experiments indicated (*n*). Data were analyzed by Student's *t*-test or one-way ANOVA and differences among groups was detected using a Dunnett's test. Statistical significance was defined as *p* < 0.05.

3. Results

3.1. GLP-1 stimulates mitochondrial fusion and function

Treatment of A7r5 cells with GLP-1 stimulated the appearance of large interconnected mitochondria (Fig. 1A). Single confocal images were used to quantify mitochondrial area and number of mitochondria per cell (Fig. 1A). GLP-1 increased mitochondrial area after 0.5 and 3 h of incubation (Fig. 1B), an effect that was reversed after 6 h. Conversely, the number of mitochondria per cell decreased significantly at 0.5 h of GLP-1 treatment (Fig. 1B). Similar data were obtained when the mitochondrial network was assessed by the number and volume of individual mitochondria through 3D reconstitution of confocal stacks (Fig. 1C). Additionally, these changes were not associated with a decrease in mitochondrial biogenesis or degradation because GLP-1 did not change the total amount of the mitochondrial protein mtHsp70 (Fig. 1D). Taken together, these results suggest that GLP-1 stimulates mitochondrial fusion in A7r5 without changing the existing balance between mitochondrial synthesis and turnover rates.

Mitochondrial fusion is associated with an increase of mitochondrial metabolism [10]. GLP-1 increased $\Delta\Psi_m$ at 0.5 h of GLP-1 treatment (Fig. 1E). Changes in ROS production by GLP-1 was observed after 6 h (Fig. 1F). To assess changes in mitochondrial oxidative phosphorylation, we measured oxygen consumption rates (OCRs) at baseline and after maximal uncoupling. Increased

basal OCR was observed after 3 h of GLP-1 treatment (Fig. 1G). Raise in OCR was due to an increased mitochondrial oxidative capacity, because CCCP-stimulated maximal OCR was significantly modified with GLP-1 treatment (Fig. 1H). Increase of $\Delta\Psi_m$ and OCR were associated with an elevation of ATP intracellular levels (Fig. 1I). These last data suggest that uncoupling among the components of the electron transport chain occurs only after reversion of mitochondrial fusion. Collectively, these data suggest that GLP-1 enhances mitochondrial activity on A7r5 cells.

3.2. GLP-1 induces Drp1 inactivation by PKA

We have recently showed that GLP-1 stimulates GLP-1 receptor/adenylate cyclase/PKA signaling pathway in VSMC [12]. To elucidate the mechanism of GLP-1-dependent mitochondrial fusion, involvement of PKA was assessed. PKA inhibition with H-89 completely inhibited mitochondrial fusion induced by GLP-1 (Fig. 2A). H-89 completely prevented the GLP-1-induced increase in mitochondrial area (Fig. 2B, upper panel) and the GLP-1-induced reduction of the number of mitochondria per cell (Fig. 2B, lower panel). Treatment with GLP-1 did not modify mitochondrial fission protein levels, Drp1 and Fis1 (Fig. 2C). However, as we described previously, GLP-1 increased mitochondrial fusion protein Mfn2, without modifying Opa1 protein levels (Fig. 2D).

Drp1 activity is inhibited by PKA phosphorylation of serine 637 and Drp1 inactivation promotes mitochondrial fusion [19]. To test whether Drp1 was responsible for GLP-1-induced mitochondrial fission, PKA-dependent Drp1 phosphorylation was assessed. GLP-1 increased Drp1 phosphorylation in serine 637. The same phosphorylation was induced by the adenylylase activator forskolin that increases intracellular cAMP levels (Fig. 2E and F). GLP-1-induced Drp1 phosphorylation was inhibited by preincubation with H-89 (Fig. 2G).

Previous studies have established the migration of Drp1 from the cytosol to the mitochondrial surface as an initial step in mitochondrial fission [10]. Therefore, we next evaluated whether mitochondrial fusion triggered by GLP-1 was associated with changes in the subcellular distribution of Drp1. Immunofluorescence studies showed that the punctuated distribution pattern of Drp1 was decreased after 3 h of treatment with GLP-1 (Fig. 2H). Moreover, GLP-1 increased the effective colocalization of Drp1 with mitochondria, but not the effective colocalization of mitochondria with Drp1, as evaluated by Manders' coefficients (Fig. 2I).

Taken together, these data suggest that GLP-1 induced Drp1 phosphorylation by an adenylylase/PKA-dependent mechanism. The decrease of Drp1 association with the mitochondria, together with an increase in Mfn2 protein levels, triggers mitochondrial fusion.

3.3. GLP-1 inhibits PDGF-BB-induced cell migration and proliferation through a PKA-dependent pathway

To test whether GLP-1 regulates A7r5 cell migration, cells were stimulated with PDGF-BB and treated with GLP-1. Treatment with GLP-1 did not modify basal A7r5 cell migration. However, pretreatment with GLP-1 prevented PDGF-BB-induced cell migration, measured by wound healing assay (Fig. 3A, upper panel). The same results were obtained using the transwell assay (Fig. 3A, lower panel). GLP-1-dependent inhibition of cell migration relies on the activation of GLP-1 receptor, because pretreatment with exendin-9 (Ex-9), a GLP-1 receptor antagonist, completely abolished the inhibitory effect of GLP-1 on PDGF-BB-induced cell migration (Fig. 3C). Forskolin mimicked the action of GLP-1 on PDGF-BB-induced cell migration (Fig. 3D). Moreover, H-89 completely abolished GLP-1 inhibitory effect on PDGF-BB-induced cell migration (Fig. 3E). Taken together, these results showed that GLP-1 inhibits

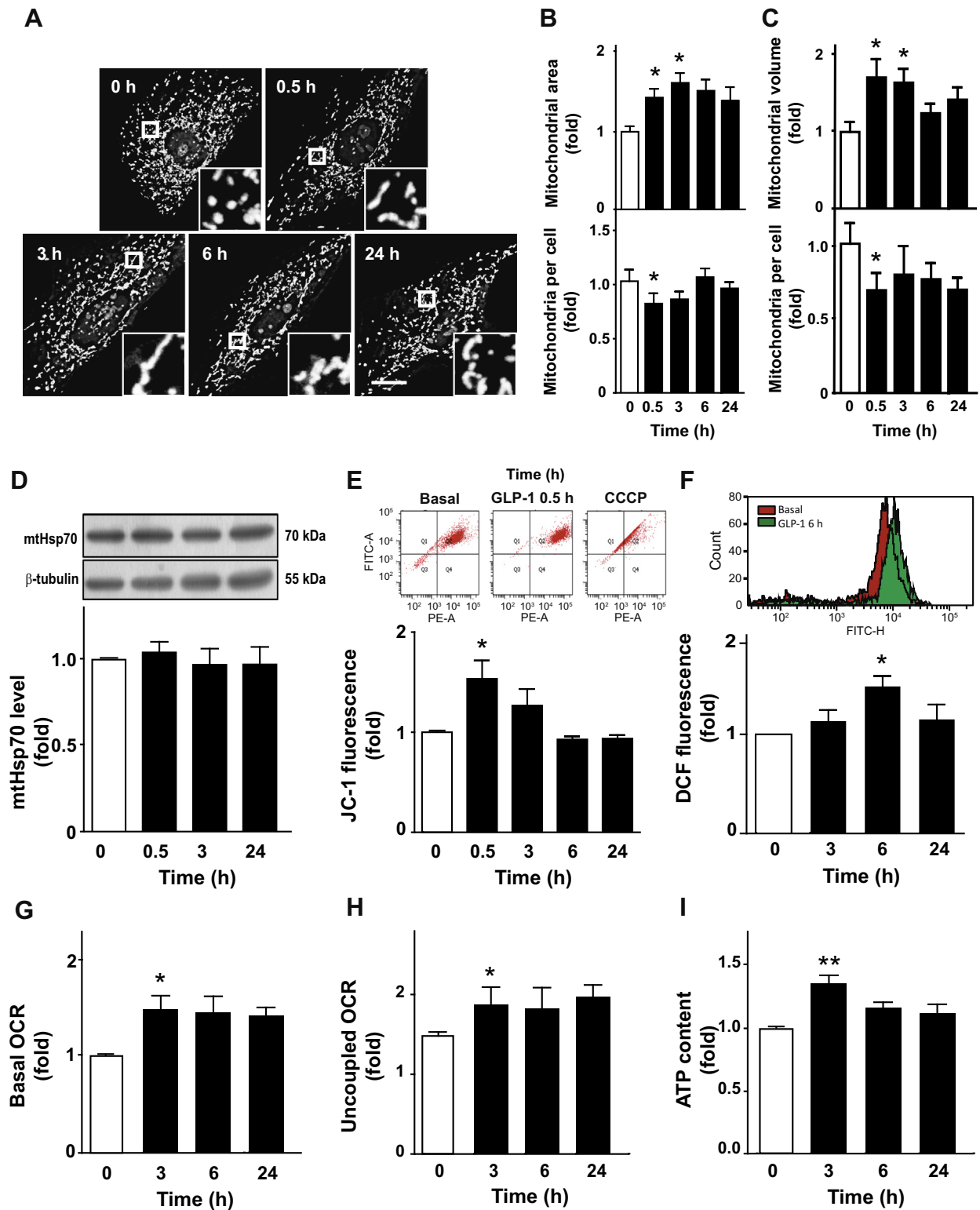


Fig. 1. GLP-1 stimulates mitochondrial fusion and activity on VSMC. A7r5 cells were stimulated with GLP-1 (100 nM) for 0, 0.5, 3, 6 and 24 h. (A) Confocal images of the mitochondrial network stained with MitoTracker Orange. Inserts are a 5× magnification of indicated areas. Scale bar = 25 μm. (B) Quantitative analysis of mitochondrial morphology. A single confocal image was used to determine mitochondrial area and number of mitochondria per cell using ImageJ software. (C) Confocal images of the mitochondrial network were subjected to 3D reconstruction to determine the volume of mitochondrial particles and the number of mitochondria per cell using image J software. (D) Total protein extracts were obtained and mtHsp70 protein levels were determined by western blot and relativized using β-tubulin. (E) Mitochondrial potential ($\Delta\Psi_m$) was determined using JC-1 probe (10 μM) and detected using a FACS system. Uncoupler CCCP (200 nM) was used as a control. Upper panel shows representative dot plots. (F) Total ROS content was measured by flow cytometry using the dihydrorhodamine (DHR) probe. Upper panel is a representative histogram of basal and A7r5 cells treated with GLP-1 for 6 h. (G) Basal oxygen consumption rate (OCR) was measured for 5 min using a Clark electrode. (H) Uncoupled respiration rate was determined by adding CCCP (200 nM). (I) ATP content was measured by a luciferin/luciferase-based kit. Data are the average \pm SEM of 4–5 independent experiments * $p < 0.05$ and ** $p < 0.01$ vs. 0 h.

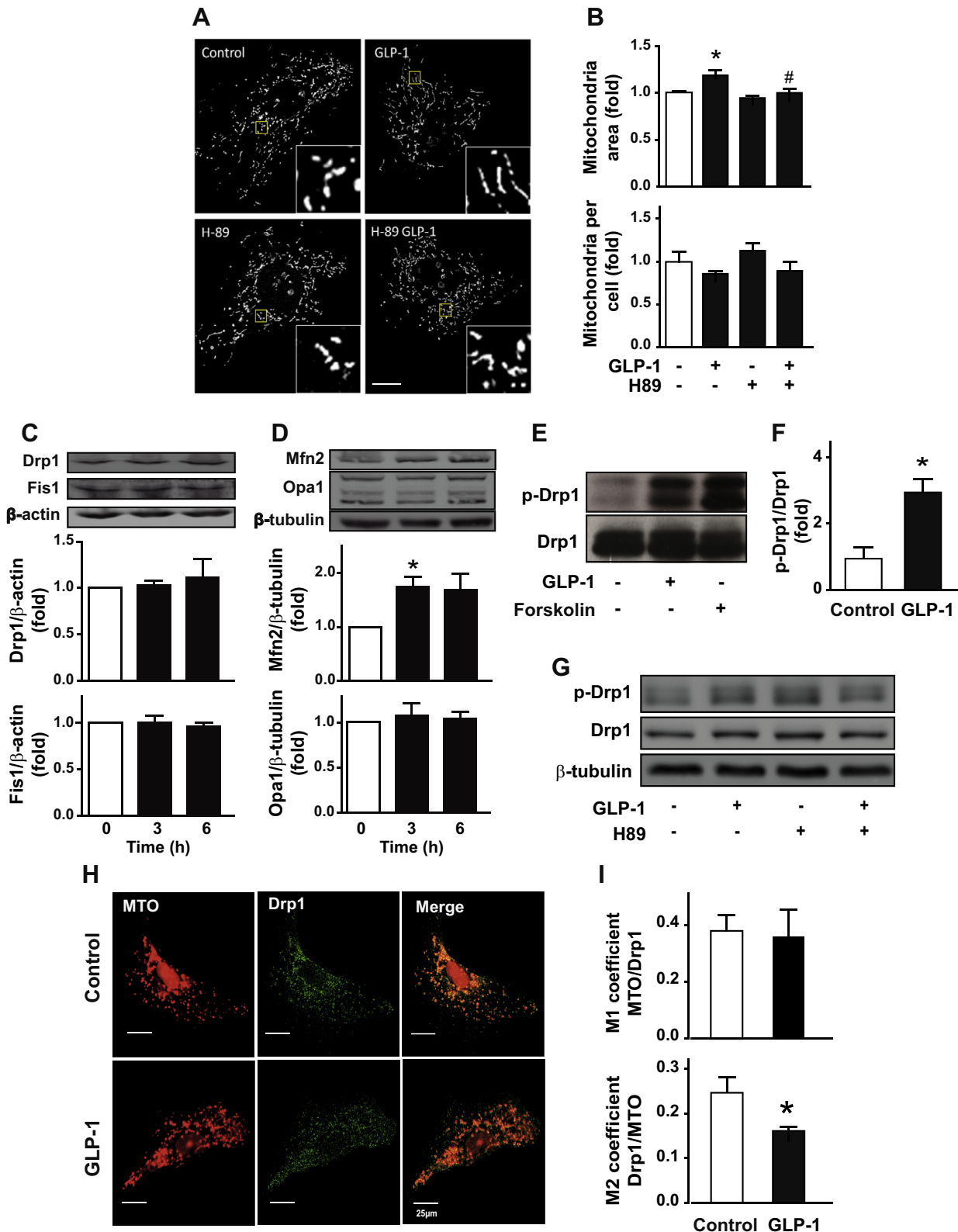


Fig. 2. GLP-1 stimulates mitochondrial fusion by a PKA/Drp1-dependent mechanism. (A) A7r5 cells were pretreated or not with H-89 (10 μM) for 30 min and then stimulated with GLP-1 (100 nM) for 3 h. Confocal images of the mitochondrial network stained with MitoTracker Orange. Inserts are a 5× magnification of indicated areas. Scale bar = 25 μm. (B) Quantitative analysis of mitochondrial morphology using the ImageJ software. Total protein extracts were obtained for A7r5 cells. (C, upper panel) Total Drp1, (C, lower panel) Fis1, (D, upper panel) Mfn2 and (D, lower panel) Opa1 protein levels were determined by western blot. β-Actin and β-tubulin were used as protein loading controls. (E) A7r5 cells were treated with GLP-1 (100 nM) for 3 h or with forskolin (100 μM). Total protein extracts were prepared and total Drp1 was immunoprecipitated as described in Section 2. Immunoprecipitated proteins were submitted to western blot using anti-p-Drp1. (F) Quantification of immunoprecipitated p-Drp1 relativized against total Drp1. (G) A7r5 cells were pretreated or not with H-89 (10 μM) for 30 min and then stimulated with GLP-1 (100 nM) for 3 h. p-Drp1 and total Drp1 was detected as described above. (H) Mitochondria and Drp1 were labeled with MitoTracker orange (MTO, 400 nM) and anti-Drp1 antibody 1:500 (green), respectively. Representative images by confocal microscopy of A7r5 cells. Bar represents 25 μm. (I) Colocalization was assessed by Manders' coefficients between mitochondria-Drp1 (upper panel) and Drp1-mitochondria (lower panel). Data are mean ± SEM of 4–5 independent experiments * *p* < 0.05 vs. control; # *p* < 0.05 vs. GLP-1. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

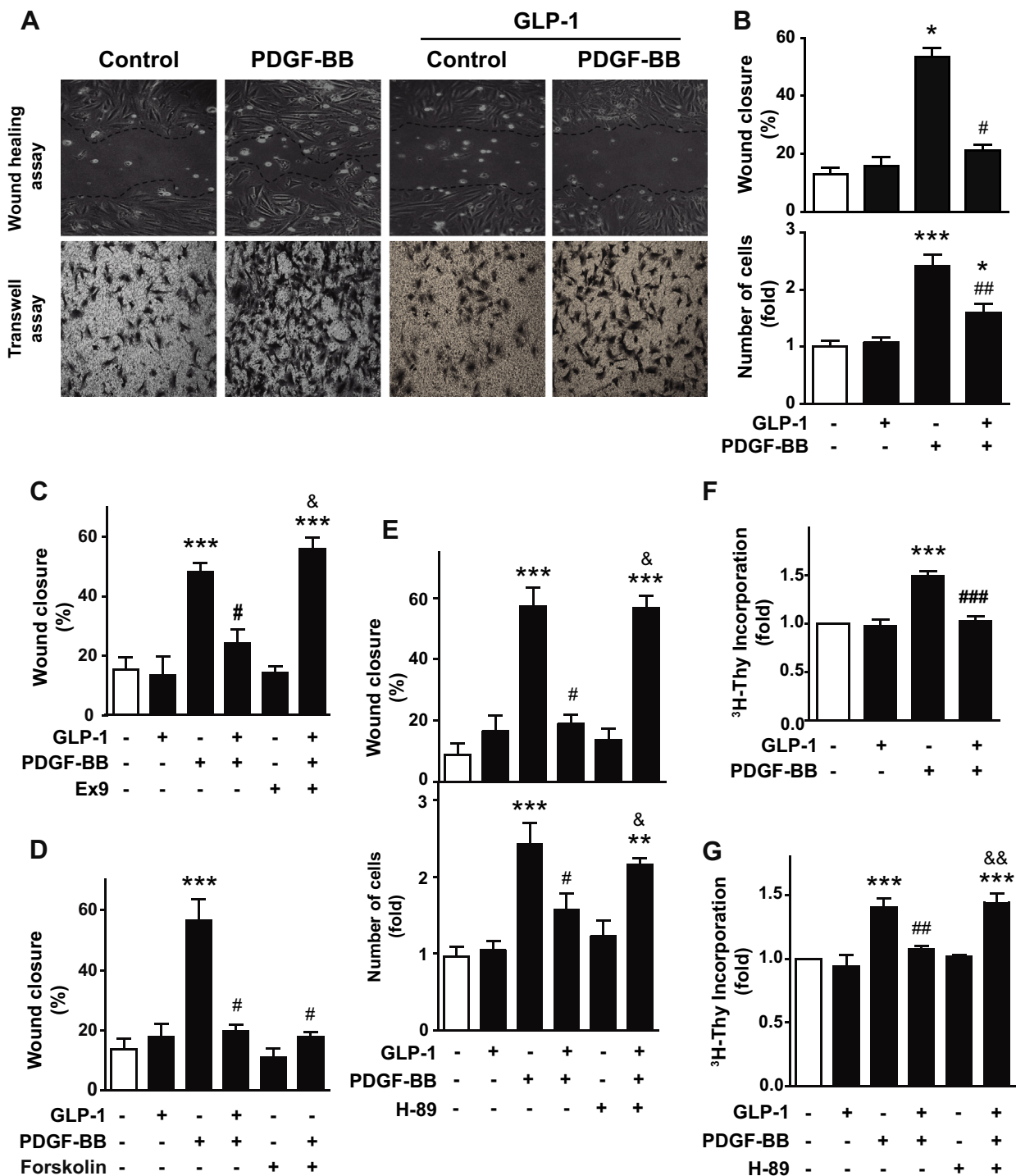


Fig. 3. GLP-1 inhibits PDGF-BB-induced cell migration and proliferation. A7r5 cells were pretreated or not with GLP-1 (100 nM) for 3 h and then stimulated with PDGF-BB (10 nM). (A) Cell migration was determined by wound healing assay (upper panel) and by transwell assay (lower panel). In the wound assay, confluent A7r5 cell monolayers were wounded by scraping and pretreated or not with GLP-1 (100 nM) for 3 h and then stimulated with PDGF-BB (10 nM) in serum-free medium. Cell migration to the wound surface was monitored from 0 to 24 h. Nine different areas of migrated cells were counted for each data point. (B, upper panel) The migrated distance of the wound edge was quantitated and expressed as % of migration with respect to the initial distance at time 0 h. (B, lower panel) In the transwell assay, cells in the lower side of the transwell membrane were stained and counted. (C) Participation of GLP-1 receptor was evaluated by incubating A7r5 cells with exendin-9 (Ex-9, 100 nM) 30 min previous to GLP-1 treatment. (D) Involvement of adenylate cyclase/cAMP was evaluated by incubating A7r5 cells with forskolin (100 μM). (E) Role of PKA in migration, assessed by wound healing assay (upper panel) and transwell assay (lower panel), was evaluated by incubating A7r5 cells with H-89 (100 nM) 30 min previous to GLP-1 or GLP-1/PDGF-BB treatments. (F) Proliferation was assessed by [³H]-thymidine incorporation. (G) Role of PKA in proliferation was evaluated by incubating A7r5 cells with H-89 (100 nM) 30 min previous to GLP-1 or GLP-1/PDGF-BB treatments. Data are mean ± SEM of 5 independent experiments. **p* < 0.05, ***p* < 0.01, ****p* < 0.001 vs. control; #*p* < 0.05, ##*p* < 0.01, ###*p* < 0.001 vs. PDGF-BB; &*p* < 0.05, &&*p* < 0.01 vs. GLP-1 + PDGF-BB.

PDGF-BB-induced cell migration through a GLP-1 receptor/adenylate cyclase/PKA pathway.

To test whether GLP-1 is involved in the control of cell proliferation, A7r5 cells were stimulated with PDGF-BB and then treated

with GLP-1. PDGF-BB but not GLP-1 induced cell proliferation, as determined by [³H]-thymidine incorporation (Fig. 3F). GLP-1-dependent inhibition of cell proliferation relies on PKA activity, because pretreatment with H-89 completely abolished GLP-1 inhibitory effect on PDGF-BB-induced cell proliferation (Fig. 3G). Taken together, these results showed that GLP-1 inhibits PDGF-BB-induced cell proliferation through a PKA-dependent mechanism.

3.4. GLP-1-dependent mitochondrial fusion inhibits PDGF-BB-induced cell migration and proliferation

PDGF-BB induced mitochondrial fission. Pretreatment with GLP-1 completely abolished PDGF-BB-induced mitochondrial fission (Fig. 4A). GLP-1 inhibited both the decrease of mitochondrial volume and the increase in the number of mitochondria per cell induced by PDGF-BB (Fig. 4B). To test whether GLP-1-dependent control of mitochondrial dynamics regulates A7r5 cell migration and proliferation, mitochondrial dynamics was manipulated by activating or inhibiting Drp1. GLP-1-dependent inhibition of PDGF-BB-induced cell migration was completely abolished by the overexpression of a wild type Drp1 (wtDrp1) (Fig. 4C, upper panel). Because Drp1 was responsible for mitochondrial fission, its overexpression induces mitochondrial fragmentation [20] antagonizing the mitochondrial fusion induced by GLP-1. The same antagonizing effect was observed when cell proliferation was assessed (Fig. 4C, lower panel). Moreover, Mdivi-1, a selective cell-permeable inhibitor of Drp1 GTPase [21,22], also inhibited PDGF-BB induced migration and proliferation (Fig. 4D). Mitochondrial fusion induced by overexpression of a domain negative Drp1 (DN-Drp1) or by overexpression of wild type Mfn2 also inhibited PDGF-BB-induced cell proliferation (Fig. 4E and F). These data suggest that mitochondrial fusion induced by GLP-1 inhibits PDGF-BB-induced cell migration and proliferation.

4. Discussion

Our findings showed for the first time that: (a) GLP-1 controls mitochondrial fusion through the regulation of Drp1 and Mfn2; and (b) GLP-1 inhibits cell migration and proliferation induced by PDGF-BB, and these effects rely on the activation of GLP-1 receptor/adenylate cyclase/PKA signaling pathway and Drp1 inhibition by a PKA-dependent phosphorylation.

In VSMC, mitochondrial dynamics is emerging as an important factor for controlling not only cell metabolism but also cell phenotype [7]. We showed that GLP-1 activates GLP-1R, stimulates PKA and induced both Drp1 phosphorylation and Mfn2 protein increase, leading to the formation of large interconnected mitochondria. Mitochondrial fusion was associated with an increase in mitochondrial activity, measured as $\Delta\Psi_m$, OCR, ATP level and ROS production.

There is a close relationship between the mitochondrial dynamics and mitochondrial metabolism [10,23]. Thus, tissues with high energy demand (i.e. skeletal muscle, cardiomyocytes, etc.) normally have fused interconnected mitochondria. Conversely, tissues with low energy requirements have smaller and fragmented mitochondria [24,25]. VSMC exhibit unusually high rates of glucose metabolism and lactate production under normal, well-oxygenated conditions [26]. Under resting conditions, the rate of oxygen consumption and lactate production is often almost equal on a molar basis, resulting in approximately 30% of the ATP supply coming from mitochondria, but at least 90% of the flux through glycolysis resulting in lactate production [27]. Therefore, VSMC have basally low mitochondrial activity that should be associated with a more fragmented mitochondrial network. Our results showed that A7r5 cells have small mitochondria, more fragmented than

we and others have described for cardiomyocytes and skeletal muscle [15,20].

GLP-1 induced a rapid mitochondrial fusion detected at 0.5–3 h of GLP-1 treatment. In accordance, $\Delta\Psi_m$, OCR and ATP levels were increased between 0.5 and 3 h after GLP-1 treatment. However, we observed that ROS was increased only after 6 h of GLP-1 incubation. Probably, this ROS increase was due to the uncoupling of mitochondrial respiratory chain due to the reversion of mitochondrial fusion induced by GLP-1. At 6 h mitochondrial morphology resembles a basal fragmented state. Moreover, at that time, these mitochondria still have increased OCR with normal $\Delta\Psi_m$. Increased electron transfer could be dissipated by ROS formation.

Our data support the idea that GLP-1-induced mitochondrial fusion depends on both an increase in Mfn2 and Drp1 inactivation by phosphorylation. Drp1 is one of the most important proteins involved in the regulation of mitochondrial fission [10,11]. Drp1 translocates from the cytosol to the mitochondria and interacts with mitochondrial outer membrane proteins (Fis-1, Caf-4, MDV-1 in yeast and Mff in metazoans) [28]. After generating a ring around the organelle, the GTP hydrolysis causes their constriction triggering the mitochondrial fission [28]. Drp1 mitochondrial translocation and GTPase activity are regulated mainly by post-translational modifications and the actin cytoskeleton [29]. Drp1 S-nitrosylation and phosphorylation by Cdk1/cyclin and PKA induce mitochondrial fission [10,30].

Our results also showed an increase in Mfn2 protein levels. We described previously that GLP-1 induced Mfn2 protein increase in a PKA-dependent mechanism [12]. As Mfn2 mRNA levels were also increased [12], it is likely that a genomic response is elicited by GLP-1. Although we previously showed that GLP-1 induced CREB phosphorylation [12], we were unable to demonstrate that CREB was responsible for Mfn2 up-regulation. Analysis of human and rat Mfn2 promoters showed that in VSMC, Mfn2 expression is regulated by Sp1 [31]. Moreover, PKA activation was reported to regulate Sp1 activity [32]. These data suggest that cAMP signaling is relevant for Mfn2 gene regulation.

Mfn2 post-translational modification by phosphorylation was first proposed by Chen et al. [33]. Zhou et al. showed that PKA-induced Ser 422 phosphorylation inhibits the anti-proliferative role of Mfn2 on VSMC [34]. However, we have shown that PKA activity is necessary for the GLP-1-dependent induction of Mfn2, effect associated with a decrease in VSMC proliferation. Zhou et al. [34] and our data seem to be controversial, but this might be just a temporally different response, as the experiments showed by Zhou et al. were conducted after 48 h of adenovirus transduction to overexpress Mfn2, while our data were obtained only after 3 h of GLP-1 stimulation. We did not test whether Mfn2 Ser422 phosphorylation was modified in response to GLP-1.

Salabei and Hill showed that PDGF-BB induces mitochondrial fragmentation in VSMC and inhibition of mitochondrial fragmentation using Mdivi-1 suppressed PDGF-BB-induced proliferation [35]. Pulmonary artery smooth muscle cell proliferation has been associated with mitochondrial fission phenotype that was also inhibited by Mdivi-1 [36]. Moreover, overexpression of Mfn2 reduced VSMC proliferation [37]. These data strongly suggest that mitochondrial fusion is associated with and inhibition of VSMC proliferation. Recently, Wang et al. showed that PDGF induced mitochondrial fission through a Drp1 dependent mechanism [38]. Moreover, they showed that PDGF induced an augmentation of mitochondrial energetics as well as ROS production, both of which were found to be necessary for VSMC migration [38]. Our data also showed that GLP-1-dependent regulation of mitochondrial dynamics controls VSMC migration and proliferation, and this mechanism relied partially on the regulation of Drp1 activity. Regulation of mitochondrial dynamics by Drp1 was previously involved in the control of human glioblastoma U251 cell migration induced by

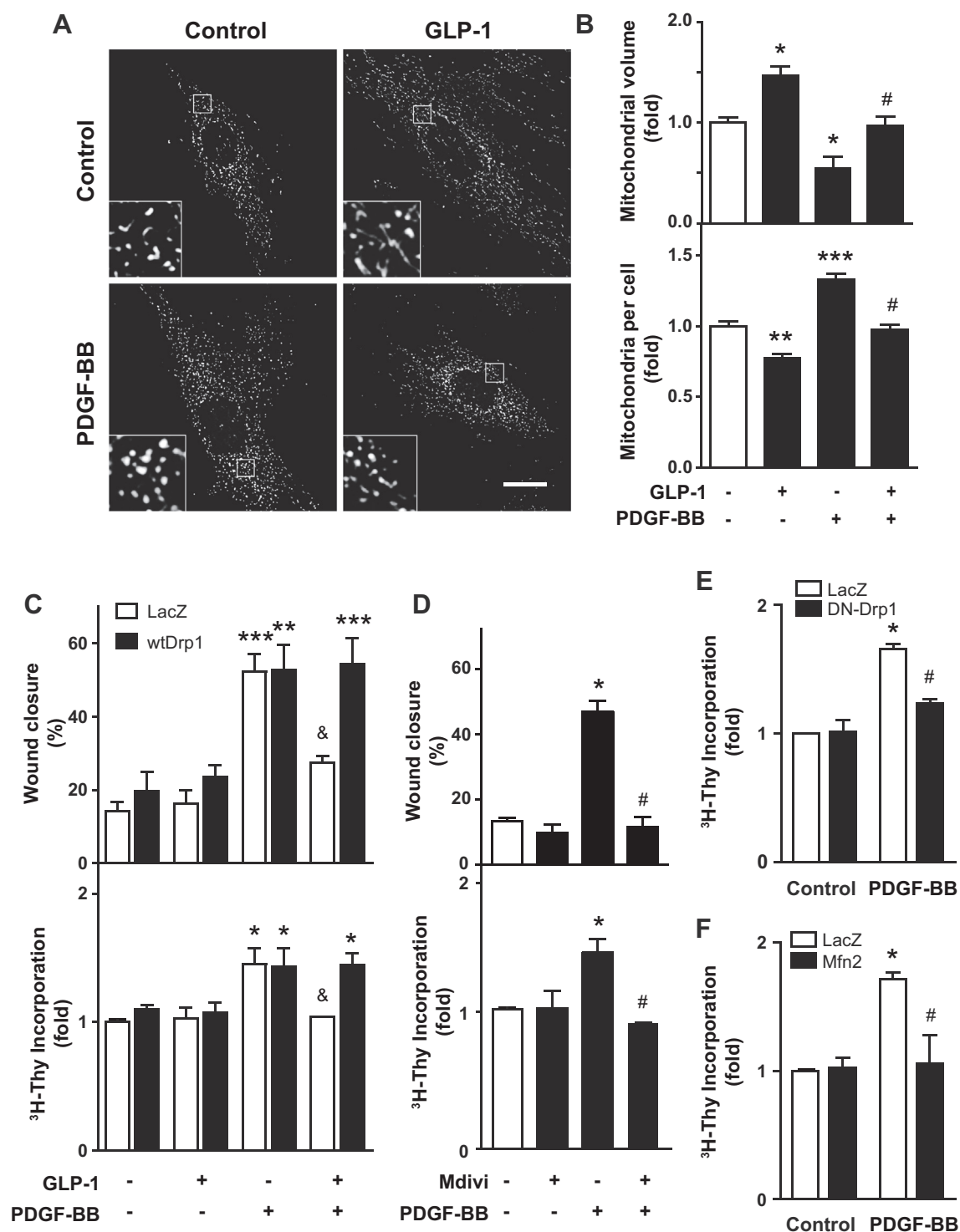


Fig. 4. GLP-1 and PDGF-BB effects on cell migration and proliferation are regulated by mitochondrial dynamics. A7r5 cells were pretreated or not with GLP-1 (100 nM) for 3 h and then stimulated with PDGF-BB (10 nM). (A) Confocal images of the mitochondrial network stained with MitoTracker Orange. Inserts are a 5 \times magnification of indicated areas. Scale bar = 25 μ m. (B) Quantitative analysis of mitochondrial morphology. (C) To assess the contribution of mitochondrial fission, A7r5 cells were transduced (MOI 300) with adenovirus overexpressing β -galactosidase (LacZ) or wild type Drp1 (wtDrp1) for 24 h prior to experiments. (D) Contribution of mitochondrial fusion was evaluated by incubating A7r5 cells with Mdivi-1 (Drp1 inhibitor, 10 μ M) 3 h previous to PDGF-BB treatment. Cell migration was determined by wound assay. Proliferation was assessed by [3 H]-thymidine incorporation. A7r5 cells were transduced with adenoviruses overexpressing β -galactosidase (LacZ, control), (E) dominant negative Drp1 (DN-Drp1) or (F) wild type Mfn2 (Mfn2) using MOI 300. Twenty-four hours later, A7r5 cells were stimulated with PDGF-BB (10 nM) and incubated with [3 H]-thymidine (1 μ Ci/mL) for 4 h. Data are mean \pm SEM of 5 independent experiments. * p < 0.05, ** p < 0.01, *** p < 0.001 vs. respective control; # p < 0.05 vs. PDGF-BB; & p < 0.05 vs. wtDrp1.

hypoxia [39] and in the migration and invasion of breast carcinoma [40].

Treatment with incretins improves fasting and postprandial lipid parameters by reducing total cholesterol, LDL-cholesterol

and triglyceride concentrations and increasing HDL cholesterol values [41]. In VSMC, incretins are able to prevent vascular remodeling [42–46]. Exendin-4 and GLP-1 prevent the progression of atherosclerosis in apoE $^{-/-}$ mice, without major effects on

metabolic parameters, and markedly reduced the accumulation of monocytes/macrophages in the vascular wall [43,45]. Moreover, in Zucker fatty rats and C57BL/6 mice, exenatide caused a significant reduction in intimal hyperplasia in balloon catheter injured carotid arteries and endothelial denudation injury of the femoral artery, respectively [42,44]. Furthermore, the GLP-1 receptor analog liraglutide significantly reduced carotid intima-media thickness, a surrogate marker of atherosclerosis, independent of glucometabolic changes, in diabetic subjects with non-alcoholic fatty liver disease [46]. These data clearly show a direct effect of GLP-1 and its analogs on the vasculature, suggesting a therapeutic role of these peptides on vascular diseases. Our results show that the vascular anti-remodeling actions of GLP-1 involve the inhibition of PDGF-BB-induced VSMC dedifferentiation through a mitochondrial dynamics-dependent mechanism.

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