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GLP-1-derived nonapeptide GLP-1(28–36)amide targets to mitochondria and suppresses glucose production and oxidative stress in isolated mouse hepatocytes

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ABSTRACT

Background: Uncontrolled hepatic glucose production (gluconeogenesis), and glycogenolysis, is a major contributor to the fasting hyperglycemia associated with type 2 diabetes. Here we report the discovery of a C-terminal nonapeptide (FIAWLVKGRamide) derived from GLP-1 that suppresses glucose production and oxidative stress in isolated mouse hepatocytes. The nonapeptide, GLP-1(28–36)amide, was reported earlier to be a major product derived from the cleavage of GLP-1 by the endopeptidase NEP 24.11.
Methods and results: Hepatocytes were isolated from the livers of normal and diet-induced obese mice. We find that the GLP-1(28–36)amide nonapeptide rapidly enters isolated mouse hepatocytes by GLP-1 receptor-independent mechanisms, and targets to mitochondria where it inhibits gluconeogenesis and oxidative stress.
Conclusions: These findings suggest that GLP-1 not only acts on a cell surface G-protein coupled receptor activating kinase-regulated signaling pathways, but a small C-terminal peptide derived from GLP-1 also enters cells, targets mitochondria, and exerts insulin-like actions by modulating oxidative phosphorylation. GLP-1(28–36)amide, or a peptide mimetic derived there from, might prove to be a useful treatment for fasting hyperglycemia and metabolic syndrome in type 2 diabetes.

1. Introduction

Glucagon-like peptide-1 (GLP-1) is a glucoincretin hormone produced in and secreted from the intestinal entero-endocrine L-cells in response to feeding [1,2]. The initial peptide cleaved from the proglucagon precursor protein is the insulinotropic peptide, GLP-1(7–36)amide that augments glucose-dependent insulin secretion. GLP-1(7–36)amide is rapidly modified in the circulation by cleavage by the diaminopeptidyl peptidase-4 (Dpp4) resulting in a loss of its insulinotropic activity [3]. Until recently Dpp4 was generally thought to degrade GLP-1(7–36)amide to an inactive metabolite, GLP-1(9–36)amide. However, the product of Dpp4 cleavage, GLP-1(9–36)amide is now believed to exert insulin-like (insulinomimetic) actions on insulin-sensitive target tissues such as the heart [4–6], cardiomyocytes [7], vasculature [8], and liver [9–12]. On heart and vasculature GLP-1(9–36)amide is reported to promote cytoprotective and anti-oxidant actions by mechanisms independent of the GLP-1 receptor [4,7–9]. These reported biochemical actions of GLP-1(9–36)amide on cells suggest that they might be exerted at the level of mitochondrial functions. GLP-1(9–36)amide was shown to lower postprandial glycemia independent of plasma insulin levels in humans [13], and to suppress hepatic glucose production both in obese, insulin-resistant subjects [9] and in isolated mouse hepatocytes in vitro [12]. Infusion of GLP-1(9–36)amide in diet-induced obese mice curtails weight gain, increases energy expenditure, and inhibits the development of diabetes and hepatic steatosis [14].

In addition to cleavages by Dpp4, GLP-1 has also been shown to be cleaved internally by the neutral endopeptidase NEP 24.11 [15]. It has been suggested that cleavages of GLP-1 by NEP 24.11 result in the degradation and the disposal of GLP-1 [16,17]. Because a major peptide product produced by the cleavage of GLP-1 by NEP 24.11 is the C-terminal nonapeptide, FIAWLVKGRamide [15], we examined the actions of this nonapeptide on isolated mouse hepatocytes. Here we report that the GLP-1(28–36)amide nonapeptide suppresses glucose production in hepatocytes and inhibits oxidative stress. Further, we find that the nonapeptide readily enters hepatocytes and targets to mitochondria as it co-localizes with the mitochondrial marker dye MitoTracker. The cellular import of GLP-1(28–36)amide occurs independently of the GLP-1 receptor. These findings suggest that GLP-1(28–36)amide directly modulates mitochondrial oxidative metabolism, such as gluconeogenesis and oxidative stress in mitochondria of hepatocytes. Because uncontrolled hepatic glucose production is a major contributor to hyperglycemia in insulin-resistant type 2 diabetes these findings raise the possibility for the development of GLP-1 nonapeptide-based small molecules for the treatment of excessive hepatic glucose production in individuals with type 2 diabetes as well as the excessive oxidative stress and insulin-resistance of the metabolic syndrome.

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2. Materials and method

2.1. Materials

MitoTracker fluorophores used were from Molecular Probes (Invitrogen, Fremont CA). The oxidized red fluorophore stains mitochondria of live and poorly respiring cells (RedCMXRos #7512) and the reduced red fluorophore stains only actively respiring cells requiring oxidation to develop fluorescence (Red CM-H2XRos #7513). The H4IIe rat hepatocyte cell line was obtained from the American Type Culture Collection (ATCC), Rockville, MD ATCC CRL 1548. All other reagents were purchased from Sigma-Aldrich, St. Louis, MO.

2.2. Peptide synthesis

GLP-1(28–36)amide [FIAWLVKGRamide] was prepared by solid phase synthesis and purified by sequential HPLC to >98% single component homogeneity. Verification of the peptides was done by both amino acid composition analysis and by mass spectroscopy.

2.3. Mice

C57bl/6J mice from 10 to 12 weeks of age were purchased from Jackson Laboratories, Bar Harbor, ME. Diet induced obesity mice (DIO) were obtained after C57bl/6J mice of 10–12 weeks of age were fed a high-fat diet (60% kcal fat, D12492, Research Diets, New Brunswick, NJ) for 9 weeks. Mice were housed and treated in accord with the regulations of the MGH Institutional Animal Care Utilization Committee.

2.4. Isolated mouse hepatocytes

Mice (male, C57bl/6) from 10 to 12 weeks of age were fasted overnight (16 h) and primary hepatocytes were isolated using a collagen and perfusion gradient purification [12]. Cells were first seeded using a Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS, 1 g/L glucose, 2 mmol/L sodium pyruvate, 1 μmol/L dexamethasone, and 0.1 μmol/L insulin, and later maintained in DMEM with 0.2% BSA, 1 g/L glucose, 0.1 μmol/L dexamethasone, and 1 nmol/L insulin.

2.5. Cultured rat H4IIe hepatoma cell line

The rat H4IIe cell line was obtained from the American Type Culture Collection, and cultured in Minimal Eagle's Medium, 5 mM glucose, supplemented with 10% fetal bovine serum, or otherwise modified as specified in the figure legends.

2.6. Mitochondrial localization

Hepatocytes were isolated from diet-induced obese (DIO) mice and plated overnight on 4 well glass slides (Nalge Nunc, Rochester NY) in maintenance medium (12). Next day cells were treated with 10 μM FAM-labeled GLP-1 (28–36) peptide (FAM-FIAWLVKGRamide) and 50 nM MitoTracker Red CM-H2Ros for 5 min in no dye, no FBS, 5 mM glucose DMEM medium. Cells were washed with the same medium and images were taken 15 min to 16 h later using Nikon Diaphot 300 inverted microscope SPOT RT camera and SPOT 3.3.1 software (Diagnostic Instruments, Sterling Heights, MI). DIO mouse liver: hepatocytes were isolated from DIO C57Bl/6J mice, plated as described above, and treated the next day for 18 h in 25 mM glucose maintenance medium with 5 μM GLP-1(28–36) and 500 nM MitoTracker Red CM-H2XRos. Cells were washed and fixed with 70% methanol/30% acetone for 30 min at room temperature. Images were captured with a Nikon Optiphot 2 microscope using Photometric Cool Snap HQ camera (Photometrics, AZ) and IP Lab4.0 software (ScanAnalytics, Inc., VA).

2.6.1. GLP-1 receptor antagonist

Hepatocytes were isolated from Ob/Ob mouse and pretreated in 25 mM glucose Krebs-Ringer buffer for 2 h with 10 μM exendin (9–39). 1 μM FAM labeled GLP-1 (28–36) and 500 nM MitoTracker Red CM-H2XRos were added into the well and images taken 30 min later were compared to the images from wells where no exendin (9–39) was used for pretreatment.

2.6.2. Irrelevant peptides

Hepatocytes isolated from Ob/Ob mice were treated for 18 h with 1 μM MitoTracker Red CM-H2XRos and 1 μM of FAM labeled GLP-1 (28–36) peptide or irrelevant peptide controls: synapsin I-derived peptide (FAM-LRRRLSDANFamide), AnaSpec Cat # 61756.

IP3R-derived peptide (FAM-GRRESLTSFGamide), AnaSpec cat. # 61731, or angiotensin I peptide (FAM-DRVYIHPFHL AnaSpec cat. # 61185), in 25 mM glucose maintenance medium. Irrelevant peptides were obtained from AnaSpec, Fremont, CA. Cells were washed with 25 mM glucose Krebs-Ringer buffer. Images were taken using Nikon Diaphot 300 inverted microscope SPOT RT camera and SPOT 3.3.1 software (Diagnostic Instruments).

2.6.3. High definition fluorescence microscopy

Hepatocytes isolated from a DIO mouse (above) were treated with MitoTracker and FAM-GLP-1(28–36)amide. After 60 min cells were visualized using a Nikon Optophot 2 and a high resolution Photometric Cool Snap HC camera and IP 4.0 software (ScanAnalytics, Sunnyvale, VA).

2.7. Confocal microscopy

Isolated mouse hepatocytes were treated with a combination of MitoTracker (red fluorescence) and FAM-GLP-1(28–36)amide (green fluorescence) for 16 h, fixed in methanol/acetone, and examined by confocal microscopy using a Nikon E800 Eclipse Radiance 2000 epifluorescence microscope with Hamamatsu Orca with a CCD camera and complete image analysis software (IP Lab). Excitation and emission spectra for red and green fluorescence were 600 nm and 650 nm, and 515 nm and 560 nm, respectively. Regions of 35–40 μm in the center of cells were recorded in twenty-seven 0.5 μm steps (2.0 μm depth).

2.8. Glucose production assay

Primary hepatocytes (2×10^5 cells per well in twelve-well plates) were pre-treated with GLP-1(28–36)amide for 1 h followed by stimulation with cAMP (10 μM)/dexamethasone (50 mM)/sodium lactate (2 mM) in glucose-free DMEM without phenol red. The culture media were collected for measuring glucose concentration with a colorimetric glucose assay kit (Sigma). The readings were then normalized to total protein content determined from whole-cell lysates.

2.9. Intracellular ATP levels

Primary hepatocytes and H4IIe cells were plated in 96-well plates at a density of 1×10^4 cells per well and treated with tert-butyl hydroperoxide (tBHP) (0.5 mM) and H_2O_2 (0.7–1.0 mM) for 1 h and overnight respectively in the presence or absence of GLP-1 (28–36) amide (100 nM). H4IIe cells were allowed to grow for 2 days before treatment. ATP levels were assessed by a ATPlite one-step luminescence ATP detection assay system (Perkin Elmer, Waltham, MA).

2.10. Reactive oxygen species (ROS) formation assay

Primary hepatocytes from diet-induced obese (DIO) and C57BL/6J mice were seeded in 96-well plates at a density of 1×10^4 cells per well for 24 h before overnight treatment with GLP-1(28–36)amide followed by the addition of tBHP (0.5 mM) for 1 h. Intracellular ROS