Original article

MOLECULAR METABOLISM

Chronic hyperglycemia downregulates GLP-1 receptor signaling in pancreatic β-cells via protein kinase A

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ABSTRACT

Objective: Glucagon-like peptide 1 (GLP-1) enhances insulin secretion and protects β-cell mass. Diabetes therapies targeting the GLP-1 receptor (GLP-1R), expressed in numerous tissues, have diminished dose-response in patients with type 2 diabetes compared with healthy human controls. The aim of this study was to determine the mechanistic causes underlying the reduced efficacy of GLP-1R ligands.

Methods: Using primary mouse islets and the β-cell line MIN6, outcomes downstream of the GLP-1R were analyzed: Insulin secretion; phosphorylation of the cAMP-response element binding protein (CREB); cAMP responses. Signaling systems were studied by immunoblotting and qRT-PCR, and PKA activity was assayed. Cell surface localization of the GLP-1R was studied by confocal microscopy using a fluorescein-tagged exendin-4 and GFP-tagged GLP-1R.

Results: Rodent β-cells chronically exposed to high glucose had diminished responses to GLP-1R agonists including: diminished insulin secretory response; reduced phosphorylation of (CREB); impaired cAMP response, attributable to chronically increased cAMP levels. GLP-1R signaling systems were affected by hyperglycemia with increased expression of mRNAs encoding the inducible cAMP early repressor (ICER) and adenylyl cyclase 8, reduced PKA activity due to increased expression of the PKA-RIα subunit, reduced GLP-1R mRNA expression and loss of GLP-1R from the cell surface. To specifically examine the loss of GLP-1R from the plasma membrane a GLP-1R-GFP fusion protein was employed to visualize subcellular localization. Under low glucose conditions or when PKA activity was inhibited, GLP-1R-GFP was found at the plasma membrane. Conversely high glucose, expression of a constitutively active PKA subunit, or exposure to exendin-4 or forskolin led to GLP-1R-GFP internalization. Mutation of serine residue 301 of the GLP-1R abolished the glucose-dependent loss of the receptor from the plasma membrane. This was associated with a loss of an interaction between the receptor and the small ubiquitin-related modifier (SUMO), an interaction that was found to be necessary for internalization of the receptor.

Conclusions: These data show that glucose acting, at least in part, via PKA leads to the loss of the GLP-1R from the cell surface and an impairment of GLP-1R signaling, which may underlie the reduced clinical efficacy of GLP-1R based therapies in individuals with poorly controlled hyperglycemia.

Keywords GLP-1 receptor; Protein kinase A; Hyperglycemia; Small ubiquitin-related modifier

1. INTRODUCTION

Incretins are peptide hormones secreted by intestinal endocrine cells in response to nutrient stimulation [1]. At the β-cells of pancreatic islets of Langerhans, incretins act to enhance insulin synthesis and secretion, reduce apoptosis and, at least in rodents, may stimulate β-cell proliferation [2]. These hormones, principally glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic peptide (GIP), bind to their Gαs-coupled receptors, GLP-1R and GIP-R, respectively, and mediate their insulinotropic and β-cell survival effects largely via increased intracellular cAMP [2,3]. This cAMP signal is transduced via the cAMP-dependent protein kinase (PKA) and the exchange proteins activated by cAMP (EPAC) [2]. Novel therapies targeting the β-cell GLP-1R and GIP-R have been introduced over the past decade to treat type 2 diabetes through the derived benefit of enhanced insulin secretion and the possibility of improved preservation of β-cell mass. Therapies targeting the GLP-1R have delivered significant sustained benefits to glucose control and β-cell function out to 4 years of treatment [4–6]. However there is a diminished β-cell response to GLP-1R agonists in pre-diabetic individuals and patients with Type 2 diabetes [7–10]. This reduced efficacy of GLP-1R agonists may involve the β-cells themselves, either through downregulation of the GLP-1R or of signaling systems lying downstream of the receptor [11]. Administration of the GLP-1R agonist, liraglutide, to young *db/db* mice with only moderate hyperglycemia provides more robust β-cell responses than in older, more hyperglycemic mice [12], indicating that hyperglycemia may be a

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contributing factor to the diminished efficacy of GLP-1 agonists in type 2 diabetes. Consistent with this, intensive insulin therapy to normalize glucose levels preceding GLP-1R administration improves the insulin secretory response in individuals with type 2 diabetes [13,14], whereas disruption of glucose homeostasis through the induction of insulin resistance diminishes the potentiating effects of GLP-1 upon insulin secretion in human subjects [15]. Understanding the mechanisms by which poorly controlled glucose diminishes GLP-1R signaling at the β-cell raises the potential for developing strategies to improve the effectiveness of GLP-1R targeting therapies. Rodent studies have shown that chronically elevated glucose downregulates both GLP-1R and GIP-R gene expression *in vivo* [16,17]. Glucose is also considered likely to activate PKC isoforms in the β-cell [18], which may be the stimulus for PKC-mediated phosphorylation of the GLP-1R that leads to its downregulation [19–21]. Homologous GLP-1R activation has been shown to downregulate the receptor rapidly [21,22], consistent with a classical negative feedback system that may be mediated via cAMP signaling. Glucose raises β-cell cAMP levels through the activation of calcium-sensitive adenylyl cyclases [23–25]. Recently we reported that hyperglycemia induces expression of the components of the SUMO (small ubiquitin-related modifier protein) pathway, which is associated with covalent modification of GLP-1R by SUMO-1 [26]. This results in downregulation of GLP1-R expression at the cell surface and impairment of GLP-1R-dependent potentiation of insulin secretion. Here we extend those findings to show that chronically elevated glucose acts via PKA to reduce GLP-1R signaling through a SUMO-1-dependent mechanism.

2. MATERIALS AND METHODS

2.1. Animals

Mice (8–10 weeks old C57BL/6J males) obtained from Harlan Laboratories and housed under conditions approved by the in the University of Chicago IACUC were used for physiological analysis and for islet isolations. Intraperitoneal glucose tolerance tests were performed in mice administered either saline or exendin-4 (at 5 μg/kg) 1 h prior to a bolus of D-glucose (2 g/kg body weight) or in mice which had received exendin-4 at 4–6 h intervals for the preceding 24 h before being administered a final dose of exendin-4 1 h prior to a bolus of D-glucose (2 g/kg body weight).

2.2. Cell culture and islet isolation and transfection

Glucose responsive early passage MIN6 cells (passage <30) used for all the experiments were grown in DMEM supplemented with 15% Fetal Bovine Serum (FBS), 100 IU/ml penicillin, and 100 mg/ml streptomycin and 3–8 (low) or 25 (high) mM glucose. Islets were isolated from 8 to 10 weeks old C57BL/6J wild-type mice (Jackson Laboratory, Bar Harbor, ME) following a protocol approved by The University of Chicago IACUC. Islets, MIN6 and isolated primary cells were transfected with Lipofectamine 2000 (Life Technologies, cat. #116688). Culture media were supplemented with exendin-4 at 10 nM (American Peptide Co. cat. # 46-3-12A), forskolin at 2 μM (Sigma–Aldrich, cat. # F6886), and H89 at 20 μM (Cell Signaling Technologies, cat # 9844). Cells were infected with adenovirus expressing an activated catalytic PKA subunit [27] or a mutated, dominant negative PKA regulatory subunit [28] to manipulate PKA activity according to a previously described protocol [29].

2.3. FRET analysis

FRET measurement of dynamic changes in cAMP was obtained in cells transfected with a plasmid expressing Epac-camps [30]. Islets were trypsinized into small primary cell clusters and cultured in RPMI 1640 medium supplemented with 10% FBS, 2 mM L-glutamine, 100 IU/ml penicillin, and 100 mg/ml streptomycin. Islet cells were co-transfected with the Epac-camps plasmid and a vector expressing monomeric red fluorescent protein driven by rat insulin 2 promoter to identify β-cells. This approach provides inefficient transfection but sufficient co-transfected cells are obtained to perform the analysis. Live cells were imaged 48 h post-transfection in KR2 buffer on a Nikon inverted epifluorescence microscope with a CCD camera. Pancreatic β-cells (Epac-camps and mRFP positive) from multiple transfections had fluorescence recorded before and during stimulation with 50 nM exendin-4. Dynamic changes in cAMP were estimated from the change in FRET ratio (acquired at 5 s intervals) by MetaFluor software (Universal Imaging) following direct addition of stimulatory glucose plus exendin-4 to the culture chamber. GLP-1R/SUMO-1 interactions were determined by live cell imaging using FRET in MIN6 cells transfected with GLP-1R-CFP or GLP-1R(S301A)-CFP and SUMO-1-YFP or SUMO-1(GG)-YFP. Cells were imaged as for analysis of cAMP. Dynamic changes in protein–protein interaction following addition of 2 μM Forskolin was observed as a decrease in FRET ratio.

2.4. Cell surface protein biotinylation

Cell surface proteins of MIN6 cells cultured for 24 h at low or high glucose were biotinylated for 45 min and purified (Thermo FisherScientific EZ-Link Sulfo-NHS-Biotinylation Kit, cat # 21425) from lysates prepared in RIPA buffer (Santa Cruz Biotechnology, cat # sc-24948). Biotinylated GFP-tagged GLP-1R receptor (membrane) and non-biotinylated receptor (cytosolic) were detected using an anti-GFP antibody (Roche Applied Science, cat # 11814460001).

2.5. ELISA and immunoblotting analyses

Insulin was quantified in the media and lysate from cells cultured under the relevant conditions for 2 h (ELISA ALPCO, Salem, NH, cat. # 80-INSMSU-E01). Cyclic AMP was quantified by ELISA (Thermo Scientific, cat # EMSCAMPL) in lysates from MIN6 cells cultured for 20 h at low (3 mM) or high (25 mM) glucose, then exposed to high glucose ± exendin-4 (10 nM) for 10 min. Immunoblotting used the following antibodies: anti-phospho-CREB (Cell Signaling Technologies, cat # 4276, diluted 1:1000); anti-total CREB (Cell Signaling Technologies, cat # 4820, diluted 1:1000); anti-PKA-RIα (Cell Signaling Technologies, cat # 5675, diluted 1:1000); anti-PKA RIIα (BD Transduction Laboratories, cat # 612242, diluted 1:1000); anti-PKA-RIIβ (BD Transduction Laboratories, cat # 610625, diluted 1:1000); anti-PKA-Cα (BD Transduction Laboratories, cat # 610980, diluted 1:1000); anti-FLAG (Cell Signaling Technologies, cat # 8146, diluted 1:500); β-catenin (Cell Signaling Technologies, cat # 9562, diluted 1:1000); anti-tubulin (Cell Signaling Technologies, cat #5346, diluted 1:2000).

2.6. Measurement of RNA levels by qRT-PCR

RNA was isolated from MIN6 cells (Qiagen RNEasy). Equimolar RNA from each sample was reverse transcribed using the First Strand cDNA Synthesis kit (Life Technologies). qPCR was performed using 20 ng of cDNA from replicate samples and the FAST SYBR Master Mix (Life Technologies). Samples were run on an iCycler with MyiQ module (BioRad). Expression was determined by comparative CT, relative to the 18S rRNA internal control (Life Technologies). Primers (Table 1) were designed using Primer3 software.

2.7. PKA activity assay

PKA activity was measured in lysates prepared from in MIN6 cells cultured for 20 h at low (3 mM) or high (25 mM) glucose using a

