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β-Arrestin1-mediated recruitment of c-Src underlies the proliferative action of glucagon-like peptide-1 in pancreatic β INS832/13 cells

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ABSTRACT

Glucagon-like peptide-1 (GLP-1), a glucoincretin hormone secreted by intestinal L cells, is a potent growth factor for the pancreatic β-cell. The development of GLP-1 mimetics and enhancers as a novel class of anti-diabetes medications underpins the importance of elucidating the molecular basis of GLP-1 signaling. In the present study, we sought to test the hypothesis that β-arrestin-mediated recruitment of c-Src underlies the proliferative action of GLP-1 in β-cells. Our results show that GLP-1 increased c-Src phosphorylation in INS832/13 cells, an effect inhibited by siRNA-mediated β-arrestin1 knockdown. Pharmacological inhibition of c-Src and overexpression of a dominant-negative c-Src mutant protein curtailed GLP-1-induced β-cell proliferation. Co-immunoprecipitation experiments showed a physical association between c-Src and both β-arrestin1 and GLP-1R upon GLP-1 treatment. Moreover, expression of β-arrestin1 mutants that lack the ability to bind c-Src blunted GLP-1-induced proliferation. Conversely, expression of a β-arrestin1 mutant that fails to target G protein-coupled receptors to clathrin-coated pits for sequestration/degradation maximally increased β-cell proliferation. We propose that the formation of a signaling complex comprising the agonist-stimulated GLP-1R, β-arrestin1 and c-Src is required for the action of GLP-1 on β-cell mass.

1. Introduction

Glucagon-like peptide-1 (GLP-1) is a glucoincretin hormone secreted by intestinal L cells. GLP-1 analogs and agents that inhibit its degradation represent a class of potent anti-diabetes medications (Drucker, 2006; Drucker and Nauck, 2006) that address both the defect in β-cell function and the decline in β-cell mass in type 2 diabetes. Thus, GLP-1 improves insulin secretion in subjects with impaired glucose tolerance and type 2 diabetes (Gutniak et al., 1992; Nauck et al., 1993), restores glucose competence in glucose-resistant β-cells (Holz et al., 1993) and enhances *insulin* gene expression as well as insulin biosynthesis (Fehmann and Habener, 1992). Finally, GLP-1 stimulates β-cell mass expansion in rodents *in vivo* and in isolated islets *ex vivo* via the promotion of β-cell proliferation (Buteau et al., 1999; Farilla et al., 2002; Stoffers et al., 2000) and survival (Buteau et al., 2004; Farilla et al., 2002; Li et al., 2003).

The GLP-1 receptor (GLP-1R) belongs to the G-protein-coupled receptor (GPCR) superfamily (Brubaker and Drucker, 2002). GLP-1R couples with Gs and activates adenylate cylcase to stimulate cAMP production. Downstream effectors of cAMP include PKA and cAMP-regulated guanine nucleotide exchange factors of the Epac family (Gromada et al., 1998; Holz et al., 1995; Kang et al., 2001). In addition, we have previously reported that GLP-1R signaling also induces proteolytic maturation of betacellulin by membrane-bound metalloproteinases to transactivate the epidermal growth factor receptor (EGFR) and stimulate β-cell proliferation (Buteau et al., 2003). In turn, EGFR sequentially activates phosphatidylinositol-3 kinase (PI3K) and Akt (Buteau et al., 2001; Buteau et al., 1999), resulting in inhibition of the forkhead transcription factor FoxO1 (Buteau et al., 2006), an effect that mediates the action of GLP-1 on β-cell proliferation and survival.

Agonist-stimulated GPCRs bind to the scaffolding proteins of the β-arrestin family. β-Arrestins not only target GPCRs for removal from the cell surface via clathrin-coated pits, but also serve as docking molecules to recruit signaling proteins such as c-Src to ligand-bound GPCRs (Luttrell et al., 1999). Our previous study suggested a possible implication of c-Src in the early steps of GLP-1 signaling (Buteau et al., 2003). However, the effects of GLP-1 on c-Src activity were not investigated and the precise molecular mechanism by which GLP-1 could activate c-Src remained unexplored. In the present study, we used an array of molecular tools to demonstrate that GLP-1 triggers the assembly of a protein complex containing GLP-1R, β-arrestin1 and c-Src. According to our model, β-arrestin1 functions as an adapter protein and recruits c-Src to the agonist-occupied receptor. We demonstrate that the

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assembly of this signaling complex is required for the proliferative action of GLP-1.

2. Materials and methods

2.1. Materials

The c-Src inhibitor, PP2, was purchased from Biomol (Plymouth Meeting, PA). Human glucagon-like peptide-1 fragment 7–36 amide was obtained from Sigma (St. Louis, MO). RPMI 1640, fetal calf serum and other culture media were purchased from Life Technologies (Burlington, ON).

2.2. Cell culture and incubation

INS832/13 (Hohmeier et al., 2000) cells (passage 36–60) were grown in RPMI-1640 medium supplemented with 10 mM HEPES, 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, 1 mM sodium pyruvate, and 50 μM β-mercaptoethanol at 37 °C in a humidified 5% CO_2 atmosphere. Cells at 70% confluence were washed with PBS and pre-incubated in serum-free RPMI supplemented with 3 mM glucose and 0.1% bovine serum albumin (BSA) (Sigma, Fraction V) 90 min prior to GLP-1 treatment.

2.3. Human islets

Human islets (purchased from Prodo Laboratories, Irvine, CA) were obtained from three donors aged between 28 and 65 years-old who died of stroke or intracranial hemorrhage. None had a history of diabetes or metabolic disorder. Approval was obtained through our institution ethics committee.

2.4. Mutant description

Plasmids for expression of wild type as well as mutant c-Src proteins were obtained from Dr. Joan Brugge (Harvard Medical School, Boston, MA). β-Arrestin1 mutants, kind gift from Dr. Robert Lefkowitz, were described before (Luttrell et al., 1999). Briefly, point mutations of β-arrestin1 on two proline residues (P91G-P121E) specifically inhibits c-Src binding without affecting GPCR sequestration. In contrast, the Val to Asp point mutation at residue 53 (V53D) inhibits GPCR sequestration and has no effect on the interaction of β-arrestin1 and c-Src. Finally, the S412D-β-arrestin1 mutant has a serine-to-aspartic acid point mutation at residue 412, which prevents both its association with c-Src and sequestration in clathrin-coated pits.

2.5. Transfection

DNA vectors were introduced into INS832/13 cells by nucleofection at a concentration of 5 μg of DNA for 6×10^6 cells. Cells were assayed the following day. For siRNA experiments, cells were transfected with either β-arrestin1 or scrambled siRNAs (Life Technologies, Burlington, ON) using Lipofactamine siRNA Max (Life Technologies, Burlington, ON) following the manufacturer's protocol.

2.6. Western blot

Proteins were extracted and quantified by BCA assay (Roche, Rockford, IL) prior to fractionation on 8 or 10% polyacrylamide gels. Anti-GLP-1R antibody was from Santa Cruz (Santa Cruz, CA). All other primary antibodies were purchased from Cell Signaling (Berverly, MA). Western blotting was performed as described before (Buteau et al., 2006).

2.7. Cell proliferation

Proliferation was evaluated using an ELISA-based BrdU incorporation kit (Roche, Indianapolis, IN). In brief, INS832/13 cells were transduced with the indicated DNA vectors, seeded in 96-well plates at 70% confluence and incubated overnight in serum-free RPMI medium supplemented with 3 mM glucose and 0.1% bovine serum albumin (BSA). BrdU was added to the culture medium for the last 1 h of the incubation period. Cells were then fixed, incubated with a peroxidase-conjugated anti-BrdU antibody and the immune complexes were quantified using a spectrophotometer to measure absorbance (Bio-Rad, Hercules, CA).

2.8. Calculations and statistics

Data are presented as means ± SEM. Statistical analyses were performed with SPSS using Student's *t*-test or ANOVA for multiple comparisons.

3. Results

3.1. c-Src mediates GLP-1 action on β-cell proliferation

Here, we sought to demonstrate that β-arrestin-mediated recruitment of c-Src is required for the action of GLP-1 on β-cell proliferation. We first investigated the effect of GLP-1 on c-Src activity by western blot. c-Src phosphorylation was evaluated after incubation of INS832/13 cells in the absence or presence of 10 nM GLP-1 for various lengths of time (Fig. 1A and B). GLP-1 induced c-Src phosphorylation in a time-dependent manner, with a maximal effect observed at 5 min. The action of GLP-1 was curtailed by PP2 (10 mM), a c-Src pharmacological inhibitor (Fig. 1C and D). We next south to confirm our result in isolated human islets. Thus, human islets were exposed to the long-lasting GLP-1 analog, exendin4, at a concentration of 10 nM for 5 min. Fig. 1E and F shows that exendin4 increased c-Src phosphorylation in normal islet tissue.

To examine whether c-Src activation is required for GLP-1-induced β-cell proliferation, we conducted BrdU incorporation measurements following GLP-1 stimulation. Incubation of INS832/13 cells with GLP-1 resulted in a 45% increase in BrdU incorporation (Fig. 1G) an effect abolished by pre-treatment of the cells with the c-Src inhibitor PP2. To further test the role of c-Src in GLP-1 action, various c-Src constructs were transfected in INS832/13 cells. We obtained a 3- to 5-fold increase in c-Src protein levels following transduction of the cells (Fig. 1H, inset). Ectopic expression of wild type or a constitutively active mutant c-Src maximally increased INS cell proliferation and this effect was non-additive to that of GLP-1 (Fig. 1H). Conversely, expression of a kinase-dead dominant-negative Src protein inhibited GLP-1-induced INS cell proliferation without significantly affecting basal proliferation. Taken together, these results suggest that c-Src activation mediates GLP-1-induced β-cell proliferation.

3.2. GLP-1 promotes the formation of a protein complex comprising GLP-1R, β-arrestin1, and c-Src

We next studied β-arrestin1 sub-cellular redistribution following GLP-1 treatment. INS832/13 cells transiently expressing β-arrestin1-GFP were stimulated with GLP-1 and the localization of β-arrestin1-GFP was investigated by microscopy (Fig. 2A). In unstimulated cells, β-arrestin1-GFP is homogeneously distributed in the cytosol. GLP-1 caused a rapid translocation of β-arrestin1-GFP and the displayed punctuate pattern suggested endosome localization, consistently with the role of β-arrestin1 in GLP-1R desensiti-