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Glucagon induces translocation of glucokinase from the cytoplasm to the nucleus of hepatocytes by transfer between 6-phosphofructo 2-kinase/fructose 2,6-bisphosphatase-2 and the glucokinase regulatory protein

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ABSTRACT

Glucokinase activity is a major determinant of hepatic glucose metabolism and blood glucose homeostasis. Liver glucokinase activity is regulated acutely by adaptive translocation between the nucleus and the cytoplasm through binding and dissociation from its regulatory protein (GKRP) in the nucleus. Whilst the effect of glucose on this mechanism is well established, the role of hormones in regulating glucokinase location and its interaction with binding proteins remains unsettled. Here we show that treatment of rat hepatocytes with 25 mM glucose caused decreased binding of glucokinase to GKRP, translocation from the nucleus and increased binding to 6-phosphofructo 2-kinase/fructose 2,6 bisphosphatase-2 (PFK2/FBPase2) in the cytoplasm. Glucagon caused dissociation of glucokinase from PFK2/FBPase2, concomitant with phosphorylation of PFK2/FBPase2 on Ser-32, uptake of glucokinase into the nucleus and increased interaction with GKRP. Two novel glucagon receptor antagonists attenuated the action of glucagon. This establishes an unequivocal role for hormonal control of glucokinase translocation. Given that glucagon excess contributes to the pathogenesis of diabetes, glucagon may play a role in the defect in glucokinase translocation and activity evident in animal models and human diabetes.
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1. Introduction

The liver plays a vital role in blood glucose homeostasis by production of glucose in the fasted state and efficient removal of glucose in the post-prandial state in response to portal hyperglycaemia for storage of glucose as glycogen or conversion to triacylglycerol [1]. Central to this process is the responsiveness of the liver to the hormone glucagon [1–3]. In normal physiology, glucagon is elevated in the post-absorptive state and acts on the liver to stimulate glucose production *via* glycogenolysis and gluconeogenesis to maintain blood glucose homeostasis [1,2,4]. After a carbohydrate-containing meal, the elevation in insulin suppresses glucagon secretion and thereby hepatic glucose production. However, in type 2 diabetes the deficiency in insulin secretion results in post-prandial hyperglucagonaemic and inadequate suppression of glucose production [2,3]. Whilst the mechanisms involved in the regulation of glycogenolysis and gluconeogenesis by glucagon have been well characterised, the effects of glucagon excess on glucose utilisation have not been fully elucidated.

Glucose metabolism by the liver is critically dependent on the activity of glucokinase, which catalyses the first-step in glucose metabolism [5,6]. Two major mechanisms are involved in the regulation of glucokinase activity: transcriptional mechanisms which account for chronic changes in protein expression [6,7] and translocation from the nucleus to the cytoplasm in response to portal hyperglycaemia or low concentrations of fructose, which accounts for the acute changes in postprandial glucose disposal [5,8].

Sequestration of glucokinase in the hepatocyte nucleus at basal glucose concentrations is regulated by binding to its inhibitory protein (GKRP) [8–10]. Stimulation with elevated concentrations of glucose (>10 mM) or micromolar concentrations of fructose or other precursors of fructose 1-phosphate causes the dissociation of the glucokinase–GKRP complex, allowing translocation of glucokinase from the nucleus

Abbreviations: BiFC, bimolecular fluorescence complementation; cAMP, cyclic adenosine monophosphate; EPAC, exchange protein directly activated by cAMP; $F26P_2$, fructose 2,6-bisphosphate; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GK, glucokinase; GKRP, glucokinase regulatory protein; G6pc, glucose 6-phosphatase; mRFP, red fluorescent protein; N/C ratio, nuclear-to-cytoplasmic ratio; PepO, desHis[1]Pro[4]Glu[9]-glucagon; PepR, desHis[1]Pro[4]Glu[9]Lys[12](γ-glutamyl PAL)glucagon-amide; Phos-a, glycogen phosphorylase; PFK2/FBPase2, 6-phosphofructo 2-kinase/fructose 2,6-bisphosphatase-2; PKA, protein kinase A; PLA, proximity ligation assay; YFP, yellow fluorescent protein

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to the cytoplasm, with consequent activation and stimulation of glycogen synthesis [5,6,8]. Various lines of evidence implicate a potential role for phosphofructo 2-kinase/fructose 2,6-bisphosphate (PFK2/FBPase2) as a cytoplasmic binding partner of glucokinase [11,12]. Whilst a role for glucose in regulating glucose translocation is well established [5,6], the role of hormones on glucokinase translocation and its interaction with PFK2/FBPase2 remains unsettled [6,13]. Compelling evidence for an over-riding role for glucagon excess in the pathogenesis of diabetes [3,14] and development of non-invasive methods for estimating glucokinase activity in man based on the assumption that glucokinase activity responds to glucose but not to hormones [15,16], calls for a critical re-evaluation of the effect of glucagon on glucokinase translocation and activity. The aims of this study were to investigate whether glucagon acutely regulates glucokinase translocation and binding to its binding partners GKRP and PFK2/FBPase2.

2. Materials and methods

2.1. Reagents

Proximity ligation assay reagents were from Olink (Uppsala, Sweden); cAMP lysis buffer was from GE healthcare (Buckinghamshire, UK); cAMP HTRF femto 2 kit was from Cisbio Bioassays (Codolet, France); Synergi C-12 column was from Phenomonex (Cheshire, UK); Site-directed mutagenesis kit was from Agilent Technologies (Berkshire, UK); NE-PER nuclear extraction kit was from Thermo Scientific (Rockford, IL); beta-actin antibody was from Sigma-Aldrich (Poole, UK); Glucokinase antibody was from Santa Cruz Biotechnology (Santa Cruz, CA); GAPDH antibody was from Hytest (Turku, Finland); Lamin A/C antibody was from New England Biolabs (Hitchin, UK); GKRP antibody was from Santa Cruz Biotechnology (Santa Cruz, CA); Total PFK2/FBPase2 antibody was from Simone Baltrusch (University of Rostock, Germany); PFK2/FBPase2 vector was from Alex Lange (University of Minnesota, Minneapolis, MN); Yellow fluorescent protein (YFP)-fragment vectors were from Tom Kerppola (University of Michigan, Ann Arbor, MI).

2.2. Hepatocyte isolation/cell culture

Hepatocytes were isolated from male Wistar rats fed *ad libitum* [12] obtained from Harlan (Bicester, UK). Procedures conformed to Home Office regulations and were approved by the local ethics committee. COS1 cells were cultured as in [17]. Cells were seeded on gelatine-coated coverslips for immunostaining, glass chambers for live-cell imaging or 24-well plates for enzyme activity, metabolite determination and flux analysis. Hepatocytes were cultured overnight in MEM in the presence of 10 nM dexamethasone and 10 nM insulin prior to treatment. All treatments were performed in the absence of dexamethasone and insulin.

2.3. Immunostaining

Hepatocyte monolayers were fixed with 4% paraformaldehyde in PBS and immunostained for glucokinase or PFK2/FBPase2 as in [12]. Nuclei were counterstained using Hoechst 33342. Cells were imaged for AlexaFluor-488 at excitation 465–495 nm; emission 515–555 nm and Hoechst at excitation 330–380 nm; emission 420 nm using a Nikon E400 microscope (×60). For method validation, 30 fields from 3 coverslips were imaged (700–950 cells). For subsequent measurements, 10 fields from 2 coverslips were imaged (80–100 cells). Fields were selected at random based on Hoechst staining. The mean pixel intensity for the nucleus and cytoplasm was analysed using Image ProPlus Software and the nuclear-to-cytoplasmic (N/C) ratio calculated for each individual cell.

2.4. Glucagon antagonists

Novel glucagon receptor antagonists desHis^{1}Pro^{4}Glu^{9}glucagon-amide (PepO) and the acylated peptide desHis^{1}Pro^{4}Glu^{9}Lys^{12}(γ-glutamyl PAL) glucagon-amide (PepR) were produced by Fmoc solid phase peptide synthesis by GL Biochem Ltd. (Shanghai, China). All peptides were >95% pure as determined by reversed-phase HPLC analysis using acetonitrile gradient elution on a Synergi C-12 column (250 × 4.6 mm). Molecular masses were checked by MALDI-TOF mass spectrometry using a Voyager-DE Biospectrometry Workstation (PerSeptive Biosytems, Framingham, MA, USA).

2.5. Proximity ligation assay (PLA)

Hepatocyte monolayers were fixed with 4% paraformaldehyde in PBS and PLA performed as in [18] using antibodies against either glucokinase and GKRP or glucokinase and PFK2/FBPase2. 20 fields from 2 to 3 coverslips/condition were selected at random based on Hoechst staining. The interaction was quantified using Blobfinder software based on either the total number of dots in the nuclear or cytoplasmic compartments or as total intensity of dots in the cell.

2.6. Enzyme activity, metabolite determination and glucose flux

Free, bound and total glucokinase activities were determined as in [12]. Free and bound glucokinase activities are expressed as percentage of total activity and total glucokinase activity expressed as mu/mg protein. Glucose phosphorylation and glycolysis were determined as in [12] and lactate production as in [19] and are expressed as nmol/h/mg protein. Glycogen phosphorylase activity was determined as in [20] and is expressed as mU/mg protein. For cAMP determination, cells were lysed using cAMP lysis buffer and cAMP concentrations quantified using the femto 2 HTRF kit as outlined by the manufacturer's instructions. cAMP concentrations are expressed as nmol/mg protein. Fructose 2,6-bisphosphate levels were determined as in [12] and are expressed as fold change relative to the absence of glucagon.

2.7. mRNA determination

Glucose 6-phosphatase (G6pc) mRNA levels were determined as described in [7]. Relative mRNA levels were calculated by the Δ cycle threshold method and were normalised to cyclophilin mRNA levels. Results were expressed relative to 5 mM glucose.

2.8. Bimolecular fluorescence complementation (BiFC)

Glucokinase-YN155, PFK2/FBPase2-YC155 and mRFP constructs were generated and the BiFC assay performed as in [17]. The serine 32 residue on the PFK2 domain was mutated to alanine (TCC to GCC) or aspartate (TCC to GAC) using site-directed mutagenesis (S32A-fwd: CGG CGA AGG GGC GCC TCC ATA CCA C; S32A-rev: GTG GTA TGG AGG CGC CCC TTC GCC G; S32D-fwd: GCA ACG GCG AAG GGG CGA CTC CAT ACC ACA GTT C; S32D-rev: GAA CTG TGG TAT GGA GTC GCC CCT TCG CCG TGG C). Sequences were confirmed by DNA sequencing and protein translation by western blotting. Constructs were co-expressed in COS1 cells and live-cell fluorescence visualised using a Nikon TE2000 fluorescence microscope (×100). Cells were imaged for yellow fluorescent protein (YFP, Excitation 500/20 nm; Emission 535/30 nm), red fluorescent protein (mRFP, Excitation 575/25 nm; Emission 632/40 nm) and Hoechst (Excitation 402/15 nm; Emission 455/20 nm). For quantification, the number of cells expressing mRFP (transfection control) and YFP (BiFC signal) was calculated (20-fields) and YFP-positive cells expressed as a percentage of mRFP-positive cells.

2.9. Nuclear fractionation

Hepatocytes were fractionated into nuclear and cytoplasmic compartments using either the NE-PER nuclear extraction kit or as in [21]. An aliquot (30 μg) of cytoplasmic or nuclear protein was subjected to SDS-PAGE following by western blotting.

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