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O-glycosylation of FoxO1 increases its transcriptional activity towards the glucose 6-phosphatase gene

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Abstract Mono-*O*-glycosylations post-translationally regulate the activity of nucleocytoplasmic proteins. We showed that glucosamine and an inhibitor of deglycosylation (PUGNAc) induced *O*-glycosylation of FoxO1, resulting in increased expression of a glucose-6-phosphatase reporter gene. This effect was independent of FoxO1 re-localisation, since it was also observed with constitutively nuclear FoxO1-AAA mutant. Moreover, in HepG2 cells, glucosamine and PUGNAc have a synergistic effect on the glucose-6-phosphatase reporter gene, and this effect was inhibited by FoxO1 siRNAs. Since glucose-6-phosphatase plays a key role in hepatic glucose production, our observation may be of importance with regard to glucotoxicity associated with chronic hyperglycaemia in diabetes.

Keywords: FoxO1; *O*-glycosylation; Glucose 6-phosphatase; Glucose toxicity; Diabetes

1. Introduction

In mammals, blood glucose concentrations are kept within narrow ranges, despite important variations in the nutritional status during the day. The importance of this regulation is underlined by the adverse consequences of chronic hyperglycaemia. Indeed, in insulin resistant patients, hyperglycaemia per se has deleterious effects (glucotoxicity) on insulin target tissues and on pancreatic β-cells, initiating a vicious circle that results in worsening of glucose intolerance and finally appearance of type 2 diabetes. Although several different mechanisms appear to be involved, mono-*O*-glycosylation of cytosolic and nuclear proteins represents an ever-growing area of research in the field [1,2]. The addition of *N*-acetylglucosamine (GlcNAc) on proteins is catalysed by *O*-linked β-*N*-acetylglucosamine transferase (OGT). OGT uses UDP-GlcNAc, produced by the hexosamine biosynthetic pathway, as a substrate to add GlcNAc on serine or threonine residues. Two to 5% of the glucose entering the cell is directed toward this pathway. Therefore, OGT can be considered as a metabolic sensor that modifies proteins according to changes in glucose availability [2]. These modifications can be rapidly reversed by β-D-*N*-acetylglucosaminidase (*O*-GlcNAcase), which removes the *O*-GlcNAc moiety from *O*-glycosylated proteins. These modifications may be particularly important in situations where glucose concentrations are altered, such as type 2 diabetes and obesity [1,2].

Excessive hepatic glucose production is a major cause of fasting hyperglycaemia in diabetes. The hydrolysis of glucose-6-phosphate by glucose-6-phosphatase (G6Pase) is the final and obligatory step for the release of glucose into the circulation. Insulin inhibits the expression of this enzyme, both in cultured hepatocytes [3] and in animals [4], resulting in inhibition of hepatic glucose production. However, most surprisingly, in cultured hepatocytes, high concentrations of glucose increased G6Pase mRNA expression [5]. In partially pancreatectomized diabetic animals, hyperglycaemia per se also increased the G6Pase mRNA expression, and this effect was reversed by phloridzin, which normalizes glycaemia independently of insulin [4].

G6Pase gene expression is known to be under the control of Forkhead Box Other-1 (FoxO1) trancription factor. Insulin, through activation of protein kinase B (PKB), induces phosphorylation of FoxO1 on serine and threonine residues, resulting in its association with 14-3-3, exclusion from the nucleus [6], decreased G6Pase expression and thereby inhibition hepatic glucose production [7]. Whereas the role of FoxO1 in the negative regulation of G6Pase expression by insulin is well established, the potential involvement of FoxO1 in glucose-induced G6Pase expression observed under chronic hyperglycaemic conditions has not been investigated yet. Since the activity of several transcription factors can be regulated by *O*-glycosylation [2,8], this work was undertaken (i) to determine whether FoxO1 is a target for *O*-glycosylation (ii) to evaluate the potential involvement of *O*-glycosylation of FoxO1 in the regulation of G6Pase expression.

2. Materials and methods

2.1. Chemicals and antibodies

All chemicals were from Sigma, except *O*-(2-acetamido-2-deoxy-D-glucopyranosylidene)amino-*N*-phenylcarbamate (PUGNAc) (Carbogen Amcis, Switzerland). Antibodies used in this work were as follows:

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Abbreviations: PUGNAc, *O*-(2-acetamido-2-deoxy-D-glucopyranosylidene)amino-*N*-phenylcarbamate; GlcN, glucosamine; GlcNAc, *N*-acetylglucosamine; OGT, *O*-linked β-*N*-acetylglucosamine transferase; *O*-GlcNAcase, β-D-*N*-acetylglucosaminidase; WGA, wheat-germ agglutinin; G6Pase, glucose 6-phosphatase

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anti-GFP (Roche Applied Science), anti-FoxO1 (H-128, Santa-Cruz), anti-*O*-GlcNAc (CTD110.6, Covance), anti-UBF (F-9, Santa-Cruz), and anti-α-tubulin (DM1A, Sigma).

2.2. Cell culture, transfection and treatments

HEK293 and HepG2 cells were maintained in DMEM containing 25 mM glucose and 10% fetal calf serum (FCS). Cells were transfected using FuGENE 6 (Roche) for cDNA or Lipofectamine 2000 (Invitrogen) for siRNAs.

Unless otherwise specified, cell treatments were performed as follows. Cells were grown in DMEM containing 5.5 mM glucose and 10% FCS. At the beginning of the treatment, this medium was replaced by the same medium containing only 1% FCS and different agents. For immunoprecipitation and wheat-germ agglutinin (WGA) precipitation, cells were treated for 24 h with either 100 μM PUGNAc or 25 mM glucose, or for 2 h with 5 mM glucosamine (GlcN). Dual-luciferase assays in HEK293 cells were performed after 24 h treatment with 100 μM PUGNAc. Luciferase assays in HepG2 cells were performed after 48 h treatment with 100 μM PUGNAc and/or 0.5 mM GlcN. For the study of mRNA expression, HepG2 cells were treated for 24 h with 100 μM PUGNAc or 0.5 mM GlcN.

2.3. Immunoprecipitation and WGA precipitation

Approximately 2×10^6 cells were lysed in buffer containing 50 mM Tris–HCl (pH 8), 137 mM NaCl, 10% (v/v) glycerol, 1% (v/v) NP40, 50 mM NaF, 10 mM di-sodium β-glycerophosphate, 1 mM Na_3VO_4, 1 mM streptozotocin and a protease inhibitor cocktail (pepstatin, antipain, leupeptin, aprotinin and AEBSF, 1 μg/ml each). For immunoprecipitation, clarified extracts (400 μg of proteins) were incubated with anti-GFP antibody and protein G-sepharose beads for 3 h at 4 °C. For WGA precipitation, cell extracts (250 μg of proteins) were incubated with 30 μl of succinylated-WGA agarose beads (Vector Laboratories) for 2 h at 4 °C. After 4 washes, proteins were eluted from the beads in Laemmli buffer, separated by SDS–PAGE and revealed by Western blotting using Enhanced Chemoluminescence (ECL, Amersham).

2.4. Luciferase assay and dual-luciferase assay

Luciferase assays (Promega) were performed using HepG2 cells stably transfected with the luciferase reporter construct containing the (−299/+57) promoter region of the human G6Pase gene (polyclonal cell line [9]). Each determination was performed in triplicate and expressed as luciferase activity per μg of protein.

Dual-luciferase assay (Promega) were performed using HEK293 cells transiently co-transfected with a plasmid containing the firefly luciferase reporter gene driven by the (−299/+57) promoter region of human G6Pase gene and a plasmid coding for Renilla luciferase to normalize for transfection efficiency. Each determination was performed in triplicate and expressed as Firefly/Renilla luciferase activities.

2.5. Statistical analysis

Statistical analysis was carried out by ANOVA using Statview 5.0 software.

3. Results

3.1. O-glycosylation of FoxO1 in HEK293 cells

To determine whether FoxO1 is subject to *O*-glycosylation, HEK293 cells transfected with CFP-tagged FoxO1 were treated with glucosamine (GlcN), which promotes *O*-glycosylation of proteins by increasing UDP-GlcNAc, and PUGNAc, which inhibits deglycosylation by *O*-GlcNAcase. *O*-glycosylated proteins were adsorbed on WGA-agarose beads and analysed by Western blotting using an anti-FoxO1 antibody (Fig. 1A). Two bands, corresponding to the molecular mass of untagged- and CFP-tagged FoxO1 (approximately 80 kDa and 110 kDa, respectively), could be detected. GlcN and PUGNAc both increased the recovery of these proteins on WGA-beads (Fig. 1A, left panel), without affecting the expression level of endogenous or transfected FoxO1 proteins (Fig. 1A, right panel). These results suggested that FoxO1 is *O*-glycosylated upon treatments that increase *O*-glycosylation or impair deglycosylation.

O-glycosylation of FoxO1 was further confirmed in immunoprecipitation experiments. HEK293 cells transfected with FoxO1-CFP were incubated with PUGNAc or GlcN. FoxO1-CFP was immunoprecipitated with an anti-GFP antibody. In the absence of PUGNAc, a basal *O*-glycosylation signal could be detected. Both PUGNAc and GlcN markedly increased *O*-glycosylation of FoxO1-CFP (Fig. 1B). Control experiments (Supplementary Fig. S1) showed that CFP alone was not *O*-glycosylated in cells treated with PUGNAc, whereas FoxO1-CFP was markedly *O*-glycosylated in the same experiments.

We also evaluated the effect of glucose on the *O*-glycosylation of FoxO1. Cells transfected with FoxO1-CFP were grown in medium containing 5.5 mM glucose for 48 h and then cultured for a further 24 h in the presence of 5.5 mM glucose, 25 mM glucose or 5.5 mM glucose plus PUGNAc (Fig. 1C). *O*-glycosylation level of FoxO1 was clearly increased in cells cultured in 25 mM glucose compared to cells maintained in 5.5 mM glucose. However, the effect of 25 mM glucose was lower than the effect of PUGNAc.

O-glycosylation can occur on regulatory phosphorylation sites of proteins, thereby controlling their activity by fine-tuning their phosphorylation on Ser or Thr. Phosphorylation of FoxO1 by PKB on Thr 24, Ser 256 and Ser 319 induces its nuclear exclusion, thereby decreasing its transcriptional activity. Replacement of these residues with alanine results in constitutive nuclear localisation and activity of the FoxO1-AAA mutant. In order to determine whether mutation of these sites impairs *O*-glycosylation of FoxO1, HEK293 cells transfected with FoxO1-AAA were incubated in the presence of PUGNAc, GlcN or high-glucose concentrations (Fig. 1D). All three treatments increased *O*-glycosylation of FoxO1-AAA, suggesting that the phosphorylation sites involved in the control of FoxO1 subcellular distribution are not major *O*-glycosylation sites. In agreement with this notion, we observed that PUGNAc did not affect the nuclear/cytosolic distribution of either wild-type or FoxO1-AAA mutant (Supplementary Fig. S2).

To determine whether *O*-glycosylation of FoxO1 regulates its transcriptional activity, HEK293 cells were transfected with a reporter gene (G6Pase-luc) constituting of the −299/+57 fragment of the human G6Pase promoter driving the firefly luciferase gene [6]. In control cells, PUGNAc had little or no effect on the luciferase activity (Fig. 2A). Co-transfection with FoxO1 resulted in higher basal and PUGNAc-induced luciferase activities. Transfection with the constitutively active FoxO1-AAA mutant resulted in a higher basal luciferase activity compared to wild-type FoxO1 (Fig. 2B), and PUGNAc further increased this activity. These results, together with those described in Fig. 1D and Supplementary Fig. S2, suggest that *O*-glycosylation of FoxO1 may play an important role in the control of G6Pase expression, independently of the phosphorylation sites that control FoxO1 cellular distribution.

Since high-glucose concentrations increased *O*-glycosylation of FoxO1, we also evaluated the effect of glucose on the G6Pase reporter-gene. We observed that in the presence of 25 mM glucose, the activity of the reporter gene was increased in FoxO1 transfected cells (Supplementary Fig. S3). However,