Role of glucose-6-phosphate and xylulose-5-phosphate in the regulation of glucose-stimulated gene expression in the pancreatic β cell line, INS-1E

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Abstract. Whether glucose-6-phosphate (G6P) or xylulose-5-phosphate (X5P) is the signaling molecule for carbohydrate response element binding protein (ChREBP) transactivation has been controversial. In this study, we tested the role of G6P and X5P in the regulation of ChREBP transactivation in the pancreatic β cell line, INS-1E. In contrast to glucose, which can be converted into both G6P and X5P, 2DG is only converted into 2DG6P. The potency of 2-deoxy-glucose (2DG) to induce Chrebp target mRNA was weaker and less persistent than that of glucose. Moreover, the results from siRNA knockdown of ChREBP, a reporter assay involving the pGL3 promoter with carbohydrate response element (ChoRE), and a ChIP assay with an anti-ChREBP antibody revealed that 2DG does not increase ChREBP transactivity in INS-1E cells. In accordance with these results, transfection of siRNA against Chrebp tended to reduce glucose-stimulated, but not 2DG-stimulated, expression of ChREBP target genes. Conversely, the expression of xylulokinase (Xylb), which converts xylitol to X5P, was much lower than in primary hepatocytes. In INS-1E cells infected by adenovirus bearing Xylb cDNA, xylitol increased expression of ChREBP target genes, although with a weaker potency than glucose. Finally, X5P partly induced ChREBP transactivity in INS-1E cells overexpressing Xylb cDNA. In conclusion, G6P and X5P can activate ChREBP transactivity, but their potencies to induce ChREBP transactivity were much lower than that of glucose, suggesting that other factors such as fructose 2,6-bisphosphate may be needed for full activation of glucose-induced gene expression.

Key words: Carbohydrate response element binding protein (ChREBP), Xylulose-5-phosphate, Glucose-6-phosphate, Xylulokinase, Thioredoxin interacting protein

EXCESS carbohydrate intake causes metabolic syndrome pathogenesis such as fatty liver, obesity, dyslipidemia, and glucose intolerance [1-3]. In metabolic syndrome, postprandial hyperglycemia and hyperinsulinemia induce hepatic de novo lipogenesis by activating insulin-activated transcription factor 1c (SREBP1c) and glucose-activated transcription factor (carbohydrate response element binding protein, ChREBP) [1]. ChREBP, also termed MondoB, is activated by glucose and regulates the expression of many genes related to glycolysis, lipogenesis, gluconeogenesis, protein secretion, and circadian rhythm [4-11]. In the liver, ChREBP is known to be activated by xylulose-5-phosphate (X5P), an intermediate metabolite in the pentose phosphate pathway. X5P activates protein phosphatase 2A (PP2A) and, in turn, PP2A dephosphorylates ChREBP to activate its transactivity [12].

In pancreatic β-cells, ChREBP regulates glucose-stimulated gene expression [13]. The pentose phosphate shuttle flux is very low in pancreatic β-cells [14]. Moreover, it has been recently proposed that glucose-6-phosphate (G6P), but not X5P, is a metabolite signal for ChREBP transactivation [15, 16]. These studies reported that G6P produced by glucokinase, but not X5P, is essential for both ChREBP nuclear translocation and transcriptional activity in response to glucose in liver cells [15]. This is a feasible idea because: (1) in ChREBP knockout mice, the hepatic G6P and glycogen content are increased [4, 5], and (2) ChREBP can regulate G6P concentration through the induction of glucose-6-phosphatase (G6pase) and glucose-6-phosphate dehydrogenase (G6pdh) gene expression [4, 5]. However, there are several problems with these
studies: (1) HepG2 cells used in these studies have much lower glucokinase activity than normal hepatocytes [17, 18], (2) in HepG2 cells, the glucose-induced gene expression between 5 and 25 mM glucose was not observed [19-21], and (3) although NADPH over-production couples with lipogenesis, lipogenesis was not increased in liver infected by adenovirus bearing G6pdh cDNA [15]. Thus, to understand the regulation of ChREBP transactivity by glucose and its metabolites, it is important to elucidate whether G6P or X5P is the glucose signaling metabolite for ChREBP transactivation [21, 22].

In this study, we investigated the contribution of glucose signals such as G6P and X5P to glucose-stimulated gene expression in INS-1E cells. Our results provide a clue to understanding the mechanism by which glucose activates ChREBP transactivity in pancreatic β-cells.

Materials and Methods

Materials and cell culture
INS-1E cells (gifted by C. B. Wollheim, University of Geneva, Switzerland) were maintained under 5% CO2 at 37°C, in RPMI-1640 medium (Invitrogen, Carlsbad, CA, USA) supplemented with penicillin and streptomycin, pyruvate, 2-mercaptoethanol, and 15% fetal calf serum [23]. 2-deoxy-glucose (2DG) and xylitol were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Measurement of glucose-6-phosphate and 2-deoxy-glucose-6-phosphate in INS-1E cells
INS-1E cells were preincubated in 10-cm dishes for 18 h in RPMI medium containing 50 mg/dL glucose, pyruvate, 2-mercaptoethanol, 15% fetal calf serum, and antibiotics. Media were then removed and cells were cultured for 2 h in RPMI medium with the indicated stimulants (2.5, 5, 10, and 25 mM glucose, or 2.5, 5, 10, and 25 mM 2-deoxy-glucose). After incubation for 2 h, cells were washed twice with ice cold PBS and 1 mL of 5% perchloric acid was added to each dish. Following neutralization with 5 mM K2CO3, cell lysates were collected for measurement of glucose-6-phosphate (G6P) and 2-deoxy-glucose-6-phosphate (2DG6P). G6P and 2DG6P were measured as previously reported except for the reaction time [24].

Plasmid construction, and adenovirus construction
The pGL3-3×TXNIP ChoRE promoter was constructed by cloning a fragment of 3×TXNIP ChoRE (cttgt-CACGAGggtgCACGAGgctccctgtgCACGAGggctgCACGAGgctcc) upstream of the SV40 promoter in the pGL3 vector. pGL3-3×PKLR ChoRE, pcDNA-daChREBP, and pRL-TK were previously described [7-11]. Rat Xylulokinase (Xylb) full length cDNA was synthesized using PrimeSTAR Max polymerase reagent (Takara Bio, Kyoto, Japan) and cloned into pENTR vector (pENTR-XYLB; Invitrogen). Using pENTR-XYLB, adenovirus bearing Xylb cDNA (Ad-XYLB) was constructed according to the manufacturer’s protocol.

RNA extraction, cDNA synthesis, and real time quantitative PCR
Cells were preincubated in RPMI-1640 media containing 2.5 mM glucose for 24 h and then cultured in RPMI-1640 media containing the indicated concentrations of glucose or 2DG for 24 h. The cells were then collected for RNA isolation and semi-quantitative real time RT-PCR analysis. INS-1E cells infected by Ad-XYLB were similarly incubated in media containing the indicated concentration of xylitol for 2 h before the cells were collected for RNA extraction and real time RT-PCR, as described previously [7, 25]. Primers used for Bhlhb2, G0s2, Gpd, Ppp1r3, Rgs16, Txinip, and Pklr were previously described [7, 25]. Primers used for rat Chrebpα were as follows: sense, 5′-CGA CACTCACCCGCCTCTTC-3′, and antisense, 5′-TT GTTCAGCCGAATCTTGTC-3′; primers used for rat Chrebpβ were: sense, 5′-TCTGCAGATCGCGCGGAG-3′, and antisense, 5′-CTTGTCCCGGCATAGCAAC-3′.

Mammalian transfection and luciferase reporter assay
INS-1E cells were cultured in 6-well plates in 2 mL of RPMI-1640 media, and preincubated in media containing 2.5 mM glucose for 24 h. For the small interfering RNA (siRNA) transfection experiments, siRNA was designed to target the coding sequence of both Chrebpα and β. The targeted sequence was 5′-UACCCUUCCUGCAGGACCAACCACAACUUU-3′ (Invitrogen). A corresponding 25-bp scrambled siRNA was used as the control. Cells were transfected with or without 100 pmols of siRNA targeting ChREBP or with the scrambled siRNA and 4 μL of Lipofectamine 2000 (Invitrogen). After 24 h of incubation in the medium containing 25 mM glucose or 25 mM 2DG, the cells
were collected and used for real time RT-PCR analysis.

For the reporter assay, cells were cultured in 6-well plates containing Lipofectamine 2000 (10 µL), pGL3-3×TXNIP ChoRE or pGL3-3×PKLR ChoRE (1.0 µg) vector, empty pcDNA or pcDNA-daChREBP (0.5 µg; da = dominant active), and pRL-TK vector (0.1 µg) [4]. After transfection with these plasmids, the cells were incubated for 24 h in media containing the indicated concentrations of the glucose analogs. Next, the cells were collected and luciferase activity was measured (Dual Luciferase Assay System; Promega, Madison, WI, USA) according to the manufacturer’s protocol.

**ChIP Assay**

The ChIP assay was performed using the EZ-Magna ChIP™ G Chromatin Immunoprecipitation Kit (Millipore, Billerica, MA, USA) according to the manufacturer’s protocol. In brief, cells were cultured in 15-cm dishes for 2 d and preincubated in RPMI-1640 media containing 2.5 mM glucose for 24 h. Cells were then cultured under 2.5 or 25 mM Glc, or 25 mM 2DG for 24 h and collected for the ChIP assay. Primer pairs were designed according to a previous study [26].

Anti-ChREBP antibody (Novus Biologicals, Littleton, CO, USA) or anti-rabbit serum (5 µg; Wako Chemical, Osaka, Japan) was used for immunoprecipitation of sonicated DNA samples. Real time PCR analysis was performed for quantification of PCR products.

**Data presentation and statistical analyses**

All data are expressed as mean ± SD. The listed n values represent the number of single experiments performed (each experiment was duplicated). Comparisons between two groups were performed using the Student’s t-test, and comparisons between multiple groups were performed using the Tukey–Kramer test. p < 0.05 was regarded as statistically significant.

**Results**

2-deoxy-glucose induces ChREBP-target genes much less than glucose

In INS-1E cells as well as primary hepatocytes, glucose is known to induce expression of ChREBP target genes in a dose-dependent manner [7-12]. To confirm that 2DG is successfully converted into 2-deoxy-glucose-6-phosphate (2DG6P), we measured 2DG6P and G6P concentration in INS-1E cells cultured in media containing 2.5, 5, 10, or 25 mM 2DG or glucose (Glc). 2DG6P concentration at 25 mM 2DG was much higher than G6P at 25 mM Glc, and we confirmed that 2DG was successfully converted to 2DG6P and accumulated in INS-1E cells (Fig. 1A). To rule out the possibility that 2DG might be harmful, we checked ATP concentration levels. At 25 mM 2DG ATP concentration levels were similar to those at 25 mM glucose (Fig. 1B). Next, we checked whether 2DG induces expression of ChREBP target genes such as basic helix-loop-helix family, member e40 (Bhlhb2), G0/G1 switch2 (G0s2), G0/S phase regulator of G-protein signaling 16 (Rgs16), Txnip, and Pklr. 2DG induced the expression of these genes much less than glucose, while adenoviral overexpression of dominant active ChREBP (daChREBP) successfully induced these genes (Fig. 1 C and 1D, respectively).

**2DG can induce Pklr and Txnip mRNA expression in a time- and dose-dependent manner in INS-1E cells**

Pklr and Txnip are well known ChREBP target genes [26, 27]. PKLR is a rate-limiting enzyme in the glycolytic pathway and plays an important role in the regulation of glycolysis and lipogenesis [6, 7]. TXNIP has an important role in glucose toxicity in pancreatic β cells [26]. In our study, 2DG and Glc increased the intracellular 2DG6P and G6P contents in INS-1E cells (Fig. 1A). We first checked whether 2DG could induce the mRNA expression of Pklr and Txnip in a dose-dependent manner similar to the intracellular 2DG6P content. After 2 h incubation with 25 mM Glc or 2DG, both Glc and 2DG increased Pklr and Txnip mRNA expression in a dose-dependent manner; however, the effect of 2DG on the induction of Pklr and Txnip gene expression was much weaker than that of Glc (Fig. 2A and 2B). Next, we checked whether Glc and 2DG affect the induction of Pklr and Txnip gene expression in a time-dependent manner. In these cells, 2DG induced both Pklr and Txnip mRNA expression in a time-dependent manner; however, the effect of 2DG on the induction of Pklr and Txnip gene expression was much smaller than that of Glc (Fig. 2C and 2D).

**2DG cannot activate ChREBP transactivity**

Next, we checked the mechanism by which 2DG induces the expression of Pklr and Txnip mRNA in INS-1E cells. First, we checked whether 2DG activates ChREBP transactivity to induce Pklr and Txnip gene expression. Transfection of siRNA against rat
ChREBP mRNA in INS-1E cells inhibited ChREBP gene expression by 50%, and tended to reduce the glucose-stimulated, but not the 2DG-stimulated, Pklr and Txnip gene expression (Fig. 3A and 3B). ChoRE was identified by Towle et al. [21], and is composed of two tandem E boxes spaced by 5 bp [28]. Glucose activates ChREBPα transactivity [27, 29]. Then, ChREBPα binds to ChoRE to induce expression of Chrebpβ and ChREBP target genes [27, 29, 30]. In agreement with Fig. 3A and 3B, a reporter assay showed that 2DG did not increase the luciferase activities of pGL3-3×PKLR or 3×TXNIP ChoRE when cotransfected with pcDNA-daChREBP or pcDNA-empty as a control; however, Glc did increase these activities in INS-1E cells (Fig. 3C and 3D). Moreover, 2DG did not increase ChREBP binding to ChoRE in INS-1E cells (Fig. 3E). Finally,
2DG by itself did not induce *Chrebpa* and *Chrebpβ* mRNA expression after 2 h incubation (Fig. 3F). Thus, we could not demonstrate that 2DG directly regulates ChREBP transactivity.

**Xylulose-5-phosphate can induce ChREBP target genes’ mRNA in INS-1E cells bearing xylulokinase mRNA**

XYLB is an enzyme that converts xylitol into xylulose-5-phosphate (Fig. 4A) [31]. Xylb is expressed in liver, however, whether Xylb is expressed in pancreatic islets is unknown [31]. We measured Xylb mRNA expression levels in INS-1E cells. Xylb mRNA in INS-1E cells was under 10% of the level in rat primary hepatocytes (Fig. 4B). Next, we tested whether xylitol induces ChREBP target genes in INS-1E cells infected by adenovirus bearing rat Xylb cDNA. In Ad-XYLB infected INS-1E cells, xylitol stimulated the mRNA expression of *Pklr*, *Txnip* and *Chrebpβ* after 7 h, although the potency of xylitol in the induction of *Pklr* and *Chrebpβ* mRNA was much weaker than that of glucose (Fig. 4C-F).

**Discussion**

X5P has been shown to be a signal for glucose-induced transactivation of ChREBP in liver [12]; however, several groups recently reported that G6P is the metabolic signal for glucose-induced ChREBP transactivity in hepatocytes and INS-1E cells [15, 16]. In this study, using 2DG, a glucose analogue that can only be converted into 2DG6P, we tested whether glucose activates ChREBP transactivity by increasing the G6P concentration in INS-1E cells. From the reporter
Fig. 3 2DG cannot activate ChREBP transactivity
(A) and (B) Transient transfection of siRNA against ChREBP caused partial inhibition of glucose-, but not 2DG-induced Pklr (A) and Txnip (B) mRNA expression. Pklr and Txnip mRNA levels were detected by real time quantitative PCR and normalized with rat Pol2. Data are presented as means ± SD (n=3) of two independent experiments. (C) and (D) 2DG did not increase the luciferase activities of pGL3-3×PKLR ChoRE (C) and 3×TXNIP ChoRE (D) when cotransfected with pcDNA-daChREBP or pcDNA-empty as control. Data are presented as means ± SD (n=6) of two independent experiments. * p < 0.05 vs. daChREBP at 2.5 mM Glc. (E) ChIP assay using anti-ChREBP antibody. (F) The effect of 2DG and glucose on Chrebpα and β mRNA levels. Chrebpα and β mRNA levels were detected by real time quantitative PCR and normalized with rat Pol2. Data are presented as means ± SD (n=3) of two independent experiments.

Fig. 4 Adenoviral overexpression of Xylulokinase cDNA causes a modest xylitol-induced expression of ChREBP target genes in INS-1E cells
(A) Schematic representation of the pathway related to xylitol metabolism. Xylulokinase converts D-Xylulose to xylulose-5-phosphate. (B) Xylulokinase mRNA levels in rat hepatocytes were much higher than in rat insulinoma cell line, INS-1E cells. (C, D, E and F) Adenoviral overexpression of rat xylulokinase cDNA caused xylitol-induced Pklr (C), Txnip (D), Chrebpα (E), and Chrebpβ (F) mRNA expression in INS-1E cells. The mRNA levels are represented as fold change compared with 2.5 mM glucose. The mRNA expression levels were analyzed by real time quantitative PCR and normalized with rat Pol2. Data are presented as means ± SD (n=3) of two independent experiments.
assay and the ChIP assay, it appears that 2DG could not increase DNA binding activity to ChoRE in INS-1E cells. Furthermore, xylitol induced a small but significant increase in expression of ChREBP target genes in Ad-XYLB infected INS-1E cells. These results suggest that both G6P and X5P partially regulate the glucose induction of ChREBP target genes in INS-1E cells as well as in rat primary hepatocytes.

The potency and duration of 2DG in inducing expression of ChREBP target genes was much weaker than that of glucose in INS-1E cells. Our results suggest that G6P cannot activate ChREBP transactivity because: (1) transient transfection of ChREBP siRNA slightly inhibited glucose, but not 2DG, induced gene expression (Fig. 3A-3B), and (2) the ChIP assay and reporter assay demonstrated that 2DG did not increase ChREBP transactivity (Fig. 3C-3E). Contrary to our evidence, a previous study reported that ChREBP siRNA completely inhibits glucose-mediated induction of Chrebp promoter activity [15]. However, these results are not consistent with those of another study showing that 3-o-methyl-glucose induces Txnip expression independently of ChREBP transactivity [32]. MondoA, a glucose-activated transcription factor, is a member of the basic helix-loop-helix leucine zipper (bHLHZIP) family of transcription factors, which bear superficial similarity to the Myc/Max/Mad family of transcription regulators. MondoA and Mlx form heterodimers to regulate expression of MondoA/Mlx target genes [32]. Moreover, 2DG and 2DG6P can increase the transactivity of MondoA. The mRNA levels of some ChREBP targets, such as Bhlhb2, Ppp1r3 and Txnip, have been shown to be increased by both 2DG and MondoA [32]. In agreement with this, 25 mM 2DG decreased the signal in the ChIP assay with anti-ChREBP antibody against Pklr and Txnip compared with 2.5 mM Glc. Moreover, adenoviral overexpression of dominant negative Mlx, which inhibits the transactivities of both ChREBP and MondoA, caused a decrease in both glucose- and 2DG-induced Pklr and Txnip expression in INS-1 cells (Fig. S1A and S1B). Together with the finding that MondoA mRNA expression is as abundant as Chrebp in INS-1E cells (Fig. S2), these results suggest that another transcription factor, probably MondoA, may compete with ChREBP on DNA binding to ChoRE. Another reason that may explain why we could not detect 2DG-induced ChREBP transactivity is the possibility that the potency of 2DG is much lower than that of glucose. We do not have an adequate antibody against MondoA, thus we cannot examine this hypothesis in the current study, however, further investigation will be needed to clarify the role of MondoA in 2DG- and 2DG6P-stimulated gene expression in INS-1E cells.

In primary hepatocytes, xylitol is converted into X5P by XYLB, and X5P enters the non-oxidative branch of the pentose phosphate pathway [31-34]. Moreover, xylitol induces the expression of ChREBP target genes such as Pklr, Sl4 and Fasn in rat primary hepatocytes [33, 34]. Thus, X5P is a glucose signal regulating the expression of glucose and lipid metabolism genes in the liver. Formerly, it has been reported that the pentose phosphate shuttle flux in pancreatic β cells is much smaller than in liver [14]. Because Xylb mRNA expression in pancreatic β cells is much lower than in liver, xylitol cannot be converted into xylulose-5-phosphate. Therefore, the pentose flux in pancreatic β cells, measured by isotope-labeled xylitol, is probably underestimated [14]. In accordance with this, xylitol successfully induced expression of ChREBP target genes in Ad-XYLB infected INS-1E cells, in which xylitol can be converted to X5P. These findings suggest that X5P can induce a small but significant expression of ChREBP target genes in INS-1E cells as well as in rat primary hepatocytes (Fig. 4B-4F).

Recently it has been reported that fructose-2,6-bisphosphate (F26P2) activates ChREBP transactivity [35]. In liver, X5P activates protein phosphatase 2A, which then activates fructose 6-phosphate 2-kinase/fructose-2,6-bisphosphatase (F6P2K) [36-38]. Finally by F6P2K, F26P2 is produced from fructose-6-phosphate [36-38]. Furthermore, fructose-2,6-bisphosphate concentration is correlated with X5P concentration in liver [39]. Therefore, we cannot rule out the possibility that F26P2 may also be a candidate ChREBP activator, although the mechanism by which F26P2 activates ChREBP transitivity is still unknown. Further investigation will be needed to clarify the interplay between X5P and F26P2 in the regulation of ChREBP.

In conclusion, our data suggest that both G6P and X5P are potential mediators regulating glucose-induced gene expression in pancreatic β-cells as well as rat primary hepatocytes. Together with the evidence that both glucose and G6P activate MondoA [30], further investigation will be needed to understand the role of G6P, X5P, and F26P2 in ChREBP and MondoA transactivation.
Acknowledgments

We thank to This work was supported by a Grant-in-Aid for Scientific Research from the Japan Society for the Promotion of Science (K. Iizuka), the Gifu University Graduate School of Medicine (K. Iizuka), the Kao Research Council for the Study of Healthcare science (K. Iizuka), Tsufu Zaidan (K. Iizuka), and the Japanese Diabetes Foundation (K. Iizuka).

Fig. S1 Adenoviral overexpression of dominant negative Mlx decreases glucose induction of Pklr and Txnip mRNA expression. Adenoviral overexpression of dominant negative Mlx (dnMlx) suppressed glucose (Glc) and 2-deoxy-glucose (2DG) induction of PKLR (A) and TXNIP (B) mRNA expression in INS-1E cells. Adenoviral overexpression of dnMlx caused about a 210-fold increase in Mlx mRNA compared with adenovirus bearing GFP cDNA. Pklr and Txnip mRNA were detected by real time quantitative PCR and normalized with rat Pol2. Data are presented as means ± SD (n=3).

Fig. S2 MondoA mRNA levels were similar to Chrebp mRNA levels in INS-1E cells. MondoA and ChREBP mRNA were detected by real time quantitative PCR and normalized with rat Pol2. Data are presented as means ± SD (n=3).

References

Glucose metabolites regulate ChREBP


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