TARGET METABOLOMICS REVEALED COMPLEMENTARY ROLES OF HEXOSE- AND PENTOSE-PHOSPHATES IN THE REGULATION OF CARBOHYDRATE-DEPENDENT GENE EXPRESSION

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Abstract

Carbohydrate response element-binding protein (ChREBP) is a transcription factor that mediates glucose signaling in mammalian liver, leading to the expression of different glycolytic and lipogenic genes, such as pyruvate kinase (L-PK) and fatty acid synthase (FAS). The current model for ChREBP activation in response to sugar phosphates holds that glucose metabolization to xylulose-5-phosphate (X5P) triggers the activation of protein phosphatase-2A, which dephosphorylates ChREBP and leads to its nuclear translocation and activation. However, evidence indicates that glucose-6-phosphate (G6P) is the most likely signal metabolite for the glucose-induced transcription of these genes. The glucose derivative that is responsible for carbohydrate-dependent gene expression remains to be identified. The difficulties in measuring G6P and X5P concentrations simultaneously and in changing them independently have hindered such identification.

To discriminate between these possibilities, we adapted a liquid chromatography mass spectrometry method to identify and quantify sugar phosphates in human hepatocarcinoma cells (HepG2) and rat hepatocytes in response to different carbon sources and in the presence/absence of a glucose-6-phosphate dehydrogenase inhibitor. We also used this method to demonstrate that these cells could not metabolize 2-deoxyglucose beyond 2-deoxyglucose-6-phosphate.

The simultaneous quantification of sugar phosphates and FAS and L-PK expression levels demonstrated that both X5P and G6P play a role in the modulation of gene expression. In conclusion, this report presents for the first time a single mechanism that incorporates the effects of X5P and G6P on the enhancement of the expression of carbohydrate-responsive genes.

KEYWORDS

Carbohydrate metabolism; Gene expression; Metabolomics; Fatty acid synthase; Pyruvate kinase.

INTRODUCTION

Glucose is the major metabolic substrate used as both energy fuel and biosynthetic precursor of several cellular components. Many cells, including hepatocytes, pancreatic β cells and adipocytes, can respond to glucose availability. Hepatocytes play a particularly important role, since the liver is the main organ responsible for maintaining an organism's energy homeostasis in response to its dietary state (25). In fasting conditions, the liver produces glucose through glycogenolysis and gluconeogenesis to supply the energy requirements of the other tissues. However, after food intake, hepatic cells convert excess carbohydrates into triglycerides through lipogenesis as a way of storing energy. These processes are coordinately regulated to utilize dietary carbohydrates efficiently (8).

Changes in key enzymes of the metabolic pathways are often mediated by altering patterns of gene expression. Therefore, it is assumed that glucose concentration may affect the gene expression of lipogenic enzymes in liver. In recent years, great progress has been achieved in understanding the molecular mechanisms that couple glucose availability to gene transcription. However, these mechanisms have not been fully characterized to date. It is known that blood glucose levels are controlled largely by glucagon and insulin produced by α and β cells in the endocrine pancreas, respectively. Insulin also regulates the transcription of lipogenic enzymes in liver through sterol regulatory element-binding protein-1c (SREBP-1c). However, elevated glucose concentrations increase the production of enzymes that are needed for *de novo* lipogenesis, such as ACC (acetyl CoA carboxylase), FAS (fatty acid synthase), and L-PK (liver pyruvate kinase) independently of SREBP-1c activity (6, 9, 10, 28). This indicates that other mechanisms are involved in the gene expression induced by carbohydrates. Glucose-responsive lipogenic genes have a DNA element in their promoters called carbohydrate response element (ChoRE) (21, 22). The ChoRE consists of two 5'-CACGTG type E box sequences separated by 5 base pairs. It serves as the recognition site for two heterodimeric transcription factors: carbohydrate response element-binding protein (ChREBP) and Max-like protein X (Mlx) (19, 23, 30). Mlx is a stable protein

that is widely expressed in glucose-responsive tissues. In contrast, ChREBP expression is highly variable in such tissues, which indicates that it is the direct target of glucose signaling (2, 7, 15).

The mechanism by which carbohydrates induce ChREBP activation is controversial. A metabolite of glucose degradation, rather than glucose itself, was found to be responsible for the glucose signal (14). Xilulose-5-phosphate (X5P), a metabolite generated by the pentose phosphate pathway (PPP) in the presence of glucose, may be involved in the process. The direct binding of X5P to protein phosphatase 2A (PP2A) stimulates its activity and subsequent ChREBP dephosphorylation, which allows the transcription factor to translocate to the nucleus and bind the ChoRE (16). Nevertheless, more recent studies have demonstrated that ChREBP dephosphorylation is not sufficient for its activation (17, 23, 27). In addition, glucose and glucose-6-phosphate (G6P) are direct agonists of liver X receptors (LXR), which are transcription factors that integrate carbohydrate and lipid metabolism in the liver. Through LXR, glucose and G6P can stimulate the expression of ChREBP and the genes involved in fatty acid synthesis (5, 13, 20).

According to the studies discussed above, two intermediate metabolites of the glucose metabolic network are the main candidates for glucose-dependent gene expression: X5P and G6P. These two molecules belong to pentose phosphate (PenP) and hexose phosphate (HexP) pools respectively. The concentration of these sugar phosphate pools inside the cells is strongly regulated, since they are involved in several metabolic pathways and are rapidly interconverted through oxidative and non-oxidative branches of the PPP. Due to this intricate metabolism, it is virtually impossible to modify G6P and X5P concentrations independently. This is one of the main problems found in studies to identify the signaling molecule. The first attempts to discriminate between X5P and G6P were designed to prevent PenP formation by using nonmetabolizable glucose analogs. The results indicated that glucose metabolism is critical for the induction of lipogenic enzymes in hepatocytes (26). Another strategy used to try to obtain cells that are rich in HexP (or in PenP) is to incubate them with PPP inhibitors and glucose (or xylitol) as an energy source. The problem with these strategies is that they result in impaired cell growth (glucose + PPP

inhibitors) or in cell death (xylitol + PPP inhibitors). This is either due to the lack of PenP, which is essential for nucleic acid synthesis, or to the lack of energetic fuel, respectively.

Furthermore, simultaneous quantification of intracellular sugar phosphate pools in mammalian cells is complex, due to their low concentration, structural similarity, instability and noncharacteristic UV absorption spectra (29). Therefore, an accurate method for sugar phosphate determination is essential to determine which molecule plays the key role in the glucose signal and to shed light in the mechanism that underlies carbohydrate-dependent gene expression.

In this study, we aimed to describe the mechanism by which sugar phosphates regulate gene expression and identify the molecular sensor involved by adapting a liquid chromatography tandem mass spectrometry (LCMS) method previously designed by our group (29). The results of the LCMS analysis allowed us to study how sugar phosphate pools vary in response to substrate incubation and, for the first time, we could correlate HexP and PenP concentrations measured simultaneously, with L-PK and FAS expression determined by real-time PCR. These data lead to the proposal of an integrative role of both G6P and X5P in the regulation of carbohydrate-dependent gene expression.

MATERIALS AND METHODS

Chemical

All chemicals were purchased from Sigma-Aldrich Co. (St. Louis MO, USA), unless otherwise specified. The media, supplements and buffers used for cell growth were purchased from Lonza (Basel, Switzerland).

Cell culture and treatment

Hepatocytes were isolated from fed rats by perfusion with collagenase in Krebs-bicarbonate buffer, following the classic perfusion/recirculation protocol (4). Cell viability as assessed by the trypan blue exclusion test was over 90%. Hepatocytes were plated at 3×10^6 cells in 6 cm tissue culture dishes in a medium containing 2.5 ml of DMEM, supplemented with 10% heat-inactivated FBS (Lonza). Four hours after seeding the cells, the medium was aspirated and the plates were washed in PBS to remove nonadherent cells. For the metabolic studies, after 24 h of preincubation in DMEM supplemented with 2% heat-inactivated FBS, 5 mM glucose, 100 nM insulin, 100 nM T3, 100 nM dexamethasone and 0.1% S/P, cells were incubated for 1, 6 or 18 h with DMEM medium (with 2% FBS, 2 mM pyruvate, 100 nM insulin, 100 nM T3, 100 nM dexamethasone and 0.1% S/P), containing either 25 mM glucose (High Glucose, HG), 5 mM glucose plus 20 mM 2-deoxyglucose (2-DOG) or 5 mM xylitol (Xyl) with or without 40 μ M dehydroepiandrosterone (DHEA). Cells without DHEA were incubated with the corresponding volume of DMSO, as this was the DHEA solvent. Finally, at the end of each manipulation, cell monolayers were immediately frozen in liquid N₂ and samples were stored at -80°C until analysis.

HepG2 cells (obtained from the American Type Culture Collection) were grown in DMEM, 25 mM D-glucose, 4 mM L-glutamine supplemented with 10% heat-inactivated FBS (PAA Laboratories, Pasching, Austria), 2 mM pyruvate and the following antibiotics: 100 U/mL penicillin and 100 μ g/mL streptomycin (0.1% S/P) (Invitrogen, Paisley, UK). They were maintained at 37°C in a humidified atmosphere containing 5% CO₂, and were passaged by trypsinization with TRIS-EDTA (0.2-0.5 M) on a weekly basis. Cells were seeded in six-well culture dishes and were preincubated in DMEM supplemented with 2% heat-inactivated FBS, 5 mM glucose, 2mM pyruvate, 100 nM insulin, 100 nM triiodothyronine (T3), 100 nM dexamethasone and 0.1% S/P. After 24 h of preincubation, cells were incubated as previously described for rat hepatocytes.

Liquid chromatography tandem mass spectrometry analysis

Hexose phosphate (HexP) and pentose phosphate (PenP) pools were determined in frozen cell monolayers of hepatocytes and HepG2 cells by liquid chromatography tandem mass spectrometry (LCMS), following the same protocol as that described by Vizan *et al.* (29). Further analyses in the API-3000 tandem triple quadrupole mass spectrometer (PE Sciex, Concord ON, Canada) with standards of 2-deoxyglucose-6-phosphate (2-DOG-6-P) and 1-deoxyxylulose-5-phosphate (1-DOX-5-P) enabled us to implement the method and determine these sugar phosphates in both cell lines. Standard addition curves were used for the quantification. The standard additions consisted of 2.0, 4.1, 8.2, 12.3, 20.5 and 32.8 μM 2-DOG-6-P; 1.2, 2.3, 4.7, 7.0, 11.7 and 18.7 μM of 1-DOX-5-P; 1.0, 1.9, 3.8, 5.8, 9.6 and 15.4 μM of glucose-6-phosphate (G6P) and, fructose-6-phosphate (F6P); and 0.5, 1.1, 2.2, 3.3, 5.4 and 8.7 μM of ribose-5-phosphate (R5P) and xylulose-5-phosphate (X5P). U- ^{13}C -glucose-6-phosphate 188 μM prepared from U- ^{13}C -glucose (Isotec Inc., Miamisburg OH, USA) was added to each sample as internal standard.

ATP/ADP quantification

ATP and ADP were extracted from cell pellets adding approximately 50 μL of 10% perchloric acid per million of cells. Cell homogenates were frozen in liquid nitrogen immediately and kept at -80°C O/N. Then extracts were thawed and incubated at 37°C for 5 min and in ice for other additional 15 min. Cell homogenates were centrifuged at 4°C (10000 rcf, 5 min) and supernatants containing nucleotides were neutralized to pH 6 with 5 M K_2CO_3 . Samples were kept at -80°C until nucleotide determination. Nucleotide levels were analyzed by injecting 15 μL of sample in a Waters Aquity UPLC system (Waters, Millford, MA). Liquid chromatography was performed on a X-Bridge^{TM} C18 column, 4.6 mm $\times$ 100 mm id, 3.5 μm particle size (Waters) with a binary gradient at a flow rate of 0.6 mL/min. The mobile phase consisted of solvent A: 0.1 M KH_2PO_4 with 4 M tetrabutylammonium hydrogen sulfate at pH 6.0 and solvent B: Metanol/solvent A

(30/70 v/v). The gradient was— 0.0 min, 0% B; 2.5 min, 0% B; 5 min, 30% B; 10 min, 40% B; 17 min, 100% B; 21 min, 100% B; 23 min, 0% B; 33 min, 0% B. The detector was programmed to read the absorbance at 260 nm. Standard mixtures for ATP and ADP quantification were used.

Protein determination

A 50 µL aliquot of sugar phosphates cell extract and of nucleotides cell homogenate was obtained as described by Vizán *et al.* (29) and the protein content was determined following the BCA protein assay (Pierce Biotechnology, Rockford IL, USA). The values were then used for the LCMS and nucleotide data normalization.

RNA extraction and gene expression determination by real-time quantitative PCR

Trizol reagent (Invitrogen) was used to isolate total RNA from cultured hepatocytes and HepG2 cells. First strand cDNA was synthesized from 1 µg of total RNA using random hexamer and expand reverse transcriptase (Roche Diagnostics, Mannheim, Germany). cDNA was used as a template for real-time PCR in a 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The relative amount of each mRNA was calculated using the second derivative comparative Ct method. The variability in the initial quantities of cDNA was normalized by using GAPDH as an endogenous control. The PCR conditions were 1 cycle at 95°C for 20 s followed by 40 cycles at 95°C for 15 s and at 60°C for 30 s. The sets of primer and TaqMan probes used to analyze cDNA were the property of Applied Biosystems (Assays-on-Demand Gene Expression Product, Applied Biosystems, Foster City, CA).

Mathematic and Statistical analyses

The principles of physics indicate that when two different variables correlate directly with a certain event, this correlation can be represented mathematically by locating both variables in the numerator of the correlation expression. In contrast, variables that have a negative effect on the event should be placed in the denominator. Since both HexP and PenP may play a positive role in the activation of carbohydrate-responding genes, variations in gene expression should correlate with the multiplication of sugar phosphate concentrations (HexP $\times$ PenP). Regression analyses were performed to corroborate the dependence of FAS and L-PK gene expression on sugar phosphate concentrations. Two linear regression models were used for each gene: M(H): $\text{Exp} = a + b \times [\text{HexP}]$; and M(H&P): $\text{Exp} = a + b \times [\text{HexP}] \times [\text{PenP}]$. In each model, a and b denote the coefficients of the straight line and Exp denotes the gene expression levels of FAS or L-PK.

Statistical significance was estimated using the Mann-Whitney U -test and Student's two-tailed t -test for unpaired observations. A p value of less than 0.05 was considered to be significant.

RESULTS

Sugar phosphate determination by liquid chromatography tandem mass spectrometry analysis

First step for the characterization of the role played by G6P and X5P in carbohydrate-dependent gene expression is to achieve the simultaneous quantification of sugar phosphate concentrations in the cell models of interest (rat hepatocytes and HepG2 cells). In order to obtain reliable data we adapted and applied a quantifiable LCMS method that was initially developed by our team to characterize sugar phosphate profiles in human adenocarcinoma HT29 cells (29). Moreover, in the current study, we used 2-DOG-6-P and 1-DOX-5-P quantification to fully characterize glucose metabolism. The m/z values for the multiple reaction monitoring (MRM) transitions for 2-DOG-6-P and 1-DOX-5-P were 243/97 and 213/97, respectively. To confirm the reliability of the method for sugar phosphate quantification in hepatocytes and HepG2 cells, we determined quality parameters, such as linearity and limits of detection as described

by Vizan and collaborators (29). The linearity ranges for each metabolite were determined according to analytical premises, to ensure the trustworthiness of the results. Specific values are depicted in Table 1. The limit of detection (LOD) and the limit of quantification (LOQ) of the method were also determined as described by Vizan and coworkers (29). Due to the clearly defined chromatographic peaks (**Fig. 1**), the method provided good limits of detection (**Table 1**). Worth to note values obtained for the main metabolites of our study in hepatocytes, HexP (0.081 μ M) and PenP (0.053 μ M).

Once the method for quantifying HexP, PenP, 2-DOG-6-P and 1-DOX-5-P had been set up, we incubated hepatocytes and HepG2 cells with different sugars as carbon and energy sources, as described in the experimental procedures. After extraction, samples were analyzed and sugar phosphates levels were measured. In rat hepatocytes, incubation with 25 mM glucose (HG) led to increases in HexP concentration, especially at 6 h of incubation. However, this did not alter the PenP concentration. In contrast, incubation with xylitol 5 mM resulted in an increase in the PenP pool as expected, as well as an unexpected increase in the HexP pool (**Fig. 2A**).

The G6PDH inhibitor, DHEA, did not induce a rise in HexP. This is not surprising given the robustness of cell metabolism and reflects that G6P is rapidly metabolized to maintain homeostasis either by glycolysis or by any pathway other than the blocked oxidative branch of PPP. Similar results were observed in HepG2 samples (data not shown).

In addition, 2-DOG-6-P was clearly synthesized in response to 2-DOG in both hepatocytes and HepG2, while its levels remained under the LOQ when it was incubated with glucose or xylitol. Using this technique, we also demonstrated that further metabolism of 2-DOG-6-P into 1-DOX-5-P was practically negligible. Thus, in incubations with 2-DOG, the concentration of 1-DOX-5-P was always very low and remained below the LOQ in every condition. These data show that the glucose analog, 2-DOG, is rapidly and efficiently metabolized by hexokinase to generate 2-DOG-6-P. Further metabolism through the

oxidative branch of PPP to produce 1-DOX-5-P is theoretically possible. However, it is not metabolically relevant in practice (concentrations of 1-DOX-5-P are always two order of magnitude lower than 2-DOG-6-P) (**Fig. 2B**).

ATP and ADP quantification

In order to confirm that the incubations with 2-DOG do not induce a dramatic decrease in the ATP/ADP ratio that could compromise some kinases activity and lead to unspecific effects over gene expression, the levels of both nucleotides were quantified and normalized by protein content in all conditions.

No difference was observed in the levels of ATP or ADP (**Fig. 3**) suggesting that incubations with 2-DOG do not affect enzymatic activity or gene expression due to alterations in the ATP/ADP ratio. Since the incubations performed never exceed 18 h cells may maintain its ATP levels thanks to their energy reserves. Therefore, metabolic and genetic effects observed in 2-DOG treated cells are not dependent on the energy state but the characteristic metabolism of the substrate.

FAS and L-PK gene expression correlates with HexP and PenP concentrations

To evaluate the correlation between HexP and PenP pools concentration and the glucose-dependent gene expression, we determined the FAS and L-PK mRNA levels, normalized by glyceraldehyde-3-phosphate dehydrogenase (GAPDH) content, by real-time PCR.

The highest expression values were observed at 18 h when 25 mM glucose was used for the incubation (9-fold for FAS and 14.5-fold for L-PK). The addition of DHEA to HG and 2-DOG conditions led to lower expression levels than the control conditions (**Fig. 4**). This effect was more noticeable in L-PK than in the FAS response. Moreover, HG conditions generated higher expression levels than Xyl and there was

a good correlation between HexP concentration and gene expression. This indicates that HexP play an important role in the gene expression activation signal pathway. Furthermore, incubation with 2-DOG did not induce FAS or L-PK expression at any time, which indicates that carbohydrate metabolism downstream of hexokinase was necessary to activate gene expression. However, DHEA caused slight decreases in HexP concentrations (**Fig. 2**). When taken together, these data suggested that HexP were necessary but not sufficient to activate FAS and L-PK expression, so more complex mechanisms may be involved. Activation of the expression of FAS and L-PK in response to sugar incubation was significantly lower in HepG2 cells than in hepatocytes. In fact, none of the conditions described in this study induced activation of L-PK expression. In addition, only weak overexpression of FAS (2- or 3-fold) was observed at 6 h for glucose and xylitol incubations, but not for 2-DOG incubation (data not shown).

Given that PenP also plays an important role in carbohydrate-dependent gene expression (16), the product of the concentrations of the two putatively involved metabolites ($\text{HexP} \times \text{PenP}$) was calculated (**Fig. 5A**). A similar profile was found when these data were compared with the gene expression levels (**Fig. 5B**). In incubations with xylitol, there were no clear correlations between ($\text{HexP} \times \text{PenP}$) and gene expression. This is not surprising if we consider that the PenP accumulated in these conditions were synthesized directly from xylitol, rather than from HexP as in the other incubation conditions.

To better discriminate whether only HexP or both HexP and PenP played a relevant role in carbohydrate-dependent gene expression, we used the two regression models described in the experimental procedures. The correlation coefficients (r^2) obtained with M(H&P) at 18 h were 0.960 for FAS and 0.987 for L-PK. At 6 h they were 0.998 for FAS and 0.979 for L-PK. However, the M(H) model showed lower correlation coefficients in three of the four adjustments and a slightly higher r^2 only for L-PK at 6 h (**Table 2**).

DISCUSSION

Carbohydrate-dependent gene expression has aroused great interest in the last three decades. Since the identification of the carbohydrate response element (ChoRE) in the promoter region of different metabolic genes (1, 9, 21, 22, 24, 26), and the characterization of carbohydrate response element-binding protein (ChREBP) as the transcription factor that is responsible for ChoRE activation (3, 30), several studies have been carried out to identify the metabolite that triggers the process. These studies have generated controversy, partly due to the lack of methods for reliably quantifying sugar phosphates, and partly because they focused on a single metabolite (11, 16, 18). Hence it is assumed that the concentrations of the other sugar phosphates varied significantly according to different incubation conditions or treatments. In this paper, we demonstrated that it is risky to assume that the concentration of sugar phosphates varies in a certain way in response to a specific treatment without experimental corroboration. The sugar phosphate metabolic network is highly interconnected and finely tuned to maintain homeostasis. Therefore, counterintuitive changes in metabolites can occasionally appear. One might think that chemical inactivation of the G6P degrading enzyme, G6PDH, with DHEA should lead to HexP accumulation; by contrast a slight decrease in HexP concentration is observed (Figure 2). This data highlights the robustness of the metabolic network and the risk of making experimentally unsubstantiated assumptions.

The LCMS method described in this paper is an innovative strategy for simultaneously quantifying HexP, PenP, 2-DOG-6-P and 1-DOX-5-P. The importance of this method lies in the fact that it constitutes the main tool for the characterization of sugar phosphates role in carbohydrate-dependent gene expression, since LCMS analysis have led us to determine the concentrations of HexP and PenP related to FAS and L-PK expression levels at every time. We showed that either HepG2 or rat hepatocytes react to incubation with different carbohydrates by balancing internal sugar phosphate concentrations. Initially, correlations between LCMS data and the expression levels of the two different carbohydrate-responding genes, FAS and L-PK, were sought in HepG2 cells. However, due to the null or weak gene overexpression obtained in

the tumor hepatic cell line in response to the different metabolic incubation conditions, we could not correlate gene expression with HexP or with HexP x PenP. In rat preneoplastic and neoplastic liver lesions, when compared with the healthy liver, the activation of insulin signaling triggers a strong induction of the AKT/mTOR cascade that is paralleled by increased synthesis of fatty acids, cholesterol, and triglycerides, induction of glycolysis, and decrease of fatty acid oxidation and gluconeogenesis (12). Interpretations based on a hepatoma cell line must be viewed with caution, and cell culture experiments cannot always mimic the specific hormonal and metabolite milieu of the liver in vivo.

Rat hepatocytes showed a more reliable and reproducible pattern of gene expression in response to sugar phosphate levels, which allowed us to demonstrate that activation of transcription cannot be justified only by modifications in the concentration of G6P. In fact, gene expression responds to both HexP and PenP pools, therefore, in addition to G6P and X5P, other metabolic species such as fructose-6-phosphate, ribose-5-phosphate and even ribulose-5-phosphate may be involved in the mechanism. Certainly, our experiments highlight the HexP as the primary signal, and the concentration of PenP as a modulating factor in the regulation of gene expression. These findings are consistent with previous studies that described G6P as a powerful stimulator of the liver X receptors (LXR). LXR are transcription factors that activate the expression of lipogenic enzymes and ChREBP in liver (20). Therefore, the main role of HexP may be due to the transcriptional effect of G6P, but once ChREBP is expressed, activation of PP2A in response to X5P concentration becomes the key to ChREBP dephosphorylation and activation (16). The mechanism proposed would imply that once the minimum concentration required to activate PP2A has been reached, further increases in X5P no longer affect gene expression levels. This would explain why PenP accumulation observed after 6 or 18 h of incubation with xylitol did not promote higher levels of FAS or L-PK expression.

In short, our data provide valuable information that can help us to elucidate orchestrated carbohydrate-dependent gene expression mechanisms. Through this mechanism, initial signaling via G6P would

promote ChREBP expression, and the subsequent carbohydrate metabolism and X5P formation would lead to the activation of the ChREBP function as a transcription factor. This increases the FAS and L-PK expression (**Fig. 6**). Such mechanisms may be influenced by many other factors such as oxidative stress, as recently suggested (31).

In conclusion, this study provides new data that allow the clarification of the respective role of G6P and X5P in the regulation of carbohydrate-dependent gene expression.

ABBREVIATIONS

1-DOX-5-P, 1-deoxyxylulose-5-phosphate; 2-DOG, 2-deoxyglucose; 2-DOG-6-P, 2-deoxyglucose-6-phosphate; ACC, acetyl CoA carboxylase; ChoRE, carbohydrate response element; ChREBP, carbohydrate response element-binding protein; DHEA, dehydroepiandrosterone; F6P, fructose-6-phosphate; FAS, fatty acid synthase; G6P, glucose-6-phosphate; HexP, hexose phosphate; HG, high glucose; LCMS, liquid chromatography tandem mass spectrometry; LOD, limit of detection; LOQ, limit of quantification; L-PK, liver-pyruvate kinase; LXR, liver X receptors; Mlx, Max-like protein; PenP, pentose phosphate; PP2A, protein phosphatase 2A; PPP, pentose phosphate pathway; R5P, ribose-5-phosphate; SREBP, sterol regulatory element-binding protein; T3, triiodothyronine; X5P, xylulose-5-phosphate; Xyl, xylitol.

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REFERENCES

1. **Bergot MO, Diaz-Guerra MJ, Puzenat N, Raymondjean M, and Kahn A.** Cis-regulation of the L-type pyruvate kinase gene promoter by glucose, insulin and cyclic AMP. *Nucleic Acids Res* 20: 1871-1877, 1992.
2. **Billin AN, Eilers AL, Queva C, and Ayer DE.** Mlx, a novel Max-like BHLHZip protein that interacts with the Max network of transcription factors. *J Biol Chem* 274: 36344-36350, 1999.
3. **Cairo S, Merla G, Urbinati F, Ballabio A, and Reymond A.** WBSCR14, a gene mapping to the Williams--Beuren syndrome deleted region, is a new member of the Mlx transcription factor network. *Human molecular genetics* 10: 617-627, 2001.
4. **Casado M, Callejas NA, Rodrigo J, Zhao X, Dey SK, Bosca L, and Martin-Sanz P.** Contribution of cyclooxygenase 2 to liver regeneration after partial hepatectomy. *Faseb J* 15: 2016-2018, 2001.
5. **Cha JY, and Repa JJ.** The liver X receptor (LXR) and hepatic lipogenesis. The carbohydrate-response element-binding protein is a target gene of LXR. *J Biol Chem* 282: 743-751, 2007.
6. **da Silva Xavier G, Rutter GA, Diraison F, Andreolas C, and Leclerc I.** ChREBP binding to fatty acid synthase and L-type pyruvate kinase genes is stimulated by glucose in pancreatic beta-cells. *J Lipid Res* 47: 2482-2491, 2006.
7. **Davies MN, O'Callaghan BL, and Towle HC.** Glucose activates ChREBP by increasing its rate of nuclear entry and relieving repression of its transcriptional activity. *J Biol Chem* 283: 24029-24038, 2008.
8. **Dentin R, Denechaud P-D, Benhamed F, Girard J, and Postic C.** Hepatic Gene Regulation by Glucose and Polyunsaturated Fatty Acids: A Role for ChREBP. *The Journal of Nutrition* 136: 1145, 2006.
9. **Dentin R, Girard J, and Postic C.** Carbohydrate responsive element binding protein (ChREBP) and sterol regulatory element binding protein-1c (SREBP-1c): two key regulators of glucose metabolism and lipid synthesis in liver. *Biochimie* 87: 81-86, 2005.
10. **Dentin R, Pegorier J-P, Benhamed F, Fougelle F, Ferre P, Fauveau V, Magnuson MA, Girard J, and Postic C.** Hepatic Glucokinase Is Required for the Synergistic Action of ChREBP and SREBP-1c on Glycolytic and Lipogenic Gene Expression. *J Biol Chem* 279: 20314-20326, 2004.
11. **Doiron B, Cuif MH, Chen R, and Kahn A.** Transcriptional glucose signaling through the glucose response element is mediated by the pentose phosphate pathway. *J Biol Chem* 271: 5321-5324, 1996.
12. **Evert M, Calvisi DF, Evert K, De Murtas V, Gasparetti G, Mattu S, Destefanis G, Ladu S, Zimmermann A, Delogu S, Thiel S, Thiele A, Ribback S, and Dombrowski F.** V-AKT murine thymoma viral oncogene homolog/mammalian target of rapamycin activation induces a module of metabolic changes contributing to growth in insulin-induced hepatocarcinogenesis. *Hepatology* 2012.

13. **Gauthier K, Billon C, Bissler M, Beylot M, Lobaccaro JM, Vanacker JM, and Samarut J.** Thyroid hormone receptor beta (TRbeta) and liver X receptor (LXR) regulate carbohydrate-response element-binding protein (ChREBP) expression in a tissue-selective manner. *J Biol Chem* 285: 28156-28163, 2010.
14. **Girard J, Ferre P, and Foulle F.** Mechanisms by which carbohydrates regulate expression of genes for glycolytic and lipogenic enzymes. *Annu Rev Nutr* 17: 325-352, 1997.
15. **Iizuka K, Bruick RK, Liang G, Horton JD, and Uyeda K.** Deficiency of carbohydrate response element-binding protein (ChREBP) reduces lipogenesis as well as glycolysis. *Proc Natl Acad Sci U S A* 101: 7281-7286, 2004.
16. **Kabashima T, Kawaguchi T, Wadzinski BE, and Uyeda K.** Xylulose 5-phosphate mediates glucose-induced lipogenesis by xylulose 5-phosphate-activated protein phosphatase in rat liver. *PNAS* 100: 5107-5112, 2003.
17. **Li MV, Chang B, Imamura M, Pongvarin N, and Chan L.** Glucose-dependent transcriptional regulation by an evolutionarily conserved glucose-sensing module. *Diabetes* 55: 1179-1189, 2006.
18. **Li MV, Chen W, Harmancey RN, Nuotio-Antar AM, Imamura M, Saha P, Taegtmeier H, and Chan L.** Glucose-6-phosphate mediates activation of the carbohydrate responsive binding protein (ChREBP). *Biochem Biophys Res Commun* 395: 395-400, 2010.
19. **Ma L, Tsatsos NG, and Towle HC.** Direct role of ChREBP.Mlx in regulating hepatic glucose-responsive genes. *J Biol Chem* 280: 12019-12027, 2005.
20. **Mitro N, Mak PA, Vargas L, Godio C, Hampton E, Molteni V, Kreusch A, and Saez E.** The nuclear receptor LXR is a glucose sensor. *Nature* 445: 219-223, 2007.
21. **Rufo C, Teran-Garcia M, Nakamura MT, Koo SH, Towle HC, and Clarke SD.** Involvement of a unique carbohydrate-responsive factor in the glucose regulation of rat liver fatty-acid synthase gene transcription. *J Biol Chem* 276: 21969-21975, 2001.
22. **Shih HM, and Towle HC.** Definition of the carbohydrate response element of the rat S14 gene. Evidence for a common factor required for carbohydrate regulation of hepatic genes. *J Biol Chem* 267: 13222-13228, 1992.
23. **Stoeckman AK, Ma L, and Towle HC.** Mlx is the functional heteromeric partner of the carbohydrate response element-binding protein in glucose regulation of lipogenic enzyme genes. *J Biol Chem* 279: 15662-15669, 2004.
24. **Thompson KS, and Towle HC.** Localization of the carbohydrate response element of the rat L-type pyruvate kinase gene. *J Biol Chem* 266: 8679-8682, 1991.
25. **Towle HC.** Glucose as a regulator of eukaryotic gene transcription. *Trends Endocrinol Metab* 16: 489-494, 2005.
26. **Towle HC, Kaytor EN, and Shih HM.** Regulation of the expression of lipogenic enzyme genes by carbohydrate. *Annu Rev Nutr* 17: 405-433, 1997.
27. **Tsatsos NG, and Towle HC.** Glucose activation of ChREBP in hepatocytes occurs via a two-step mechanism. *Biochem Biophys Res Commun* 340: 449-456, 2006.
28. **Vaulont S, Vasseur-Cognet M, and Kahn A.** Glucose regulation of gene transcription. *J Biol Chem* 275: 31555-31558, 2000.
29. **Vizan P, Alcarraz-Vizan G, Diaz-Moralli S, Rodriguez-Prados JC, Zanuy M, Centelles JJ, Jauregui O, and Cascante M.** Quantification of Intracellular Phosphorylated Carbohydrates in HT29 Human Colon Adenocarcinoma Cell Line Using Liquid Chromatography-Electrospray Ionization Tandem Mass Spectrometry. *Anal Chem* 79: 5000-5005, 2007.

30. Yamashita H, Takenoshita M, Sakurai M, Bruick RK, Henzel WJ, Shillinglaw W, Arnot D, and Uyeda K. A glucose-responsive transcription factor that regulates carbohydrate metabolism in the liver. *Proc Natl Acad Sci U S A* 98: 9116-9121, 2001.
31. Yu FX, Chai TF, He H, Hagen T, and Luo Y. Thioredoxin-interacting protein (Txnip) gene expression: sensing oxidative phosphorylation status and glycolytic rate. *J Biol Chem* 285: 25822-25830, 2010.

FIGURES

FIGURE 1. Limits of detection for sugar phosphate concentration by LCMS method. Multiple reaction monitoring (MRM) chromatograms were obtained from cellular extracts with 3 ng/μL glucose-6-phosphate and fructose-6-phosphate (HexP), 1.5 ng/μL ribose-5-phosphate and xylulose-5-phosphate (PenP), 5 ng/μL 2-deoxyglucose-6-phosphate (2-DOG-6-P) or 2.5 ng/μL 1-deoxyxylulose-5-phosphate (1-DOX-5-P). (A) Hepatocytes extract sample and (B) HepG2 extract sample.

FIGURE 2. LCMS analysis of sugar phosphates. Rat hepatocytes were incubated for 0 to 18 hours with 25 mM glucose (HG), 20 mM 2-deoxyglucose plus 5 mM glucose (2-DOG) or 5 mM xylitol (Xyl) in the absence (Ct) or presence (DHEA) of 40 μM dehydroepiandrosterone. The variations in hexose phosphate (HexP), pentose phosphate (PenP), 2-deoxyglucose-6-phosphate (2-DOG-6-P) and 1-deoxyxylulose-5-phosphate (1-DOX-5-P) concentrations were measured in hepatocyte extracts by LCMS. Results are the mean ± SE from three separate experiments.

FIGURE 3. Quantification of nucleotide phosphates. Rat hepatocytes were incubated for 0 to 18 hours with 25 mM glucose (HG), 20 mM 2-deoxyglucose plus 5 mM glucose (2-DOG) or 5 mM xylitol (Xyl) in the absence (Ct) or presence (DHEA) of 40 μM dehydroepiandrosterone. Nucleotides were extracted after treatment and ATP and ADP were quantified by UPLC-UV analysis. ATP and ADP concentrations were normalized by protein content. Results are expressed as mean ± SE from three separate experiments.

FIGURE 4. q-PCR analysis of FAS and L-PK mRNA levels. Rat hepatocytes were incubated with 25 mM glucose (HG), 20 mM 2-deoxyglucose plus 5 mM glucose (2-DOG) or 5 mM xylitol (Xyl) in the absence (Ct) or presence (DHEA) of 40 μ M dehydroepiandrosterone. Total RNA was extracted after treatment and cDNA was synthesized by reverse transcriptase. The amount of FAS or L-PK expression was determined in each condition considering the basal condition (Ct at 0h) as reference value (scaled in arbitrary units, a.u.). Results are the mean $\pm$ SE from three separate experiments.

FIGURE 5. Profile of (HexP X PenP) pools and correlation with gene expression. **A.** (HexP X PenP) products were calculated by multiplying hexose-P and pentose-P concentrations in response to incubation with 25 mM glucose (HG), 20 mM 2-deoxyglucose plus 5 mM glucose (2-DOG) or 5 mM xylitol (Xyl) in the absence (Ct) or presence (DHEA) of 40 μ M dehydroepiandrosterone. Differences between Ct (0h) and the rest of the conditions are depicted (*, $p < 0.05$; **, $p < 0.01$). **B.** (HexP X PenP) products were correlated with L-PK and FAS expression in all conditions.

FIGURE 6. Hypothetical mechanism showing the activation of carbohydrate-dependent genes in response to G6P and X5P. G6P induces ChREBP expression through LXR activity. ChREBP is initially phosphorylated and inactive. On the other hand X5P induces enzyme PP2A activation which in turn dephosphorylates ChREBP leading to its nuclear localization and its transcription factor function. Inactive enzymes are represented as solid grey figures and active enzymes as white and grey ones. Different arrows connecting G6P and X5P represent every reaction in the pentose phosphate pathway. G6P: glucose-6-phosphate, X5P: xylulose-5-phosphate, LXR: liver X receptor, ChREBP: carbohydrate response element-binding protein, PP2A: protein phosphatase 2A, FAS: fatty acid synthase, L-PK: liver pyruvate kinase.

TABLES

TABLE 1. Quality parameters of the LCMS method LOD, Limit of detection; LOQ, limit of quantification; G6P, glucose-6-phosphate; F6P, fructose-6-phosphate; R5P, ribose-5-phosphate; X5P, xylulose-5-phosphate; 2-DOG-6-P, 2-deoxyglucose-6-phosphate; 1-DOX-5-P, 1-deoxyxylulose-5-phosphate

TABLE 2. Correlation coefficients (r^2) shown by the regression models ^a Linear regression model that correlates gene expression with the product of hexose phosphate by pentose phosphate concentrations. ^b Linear regression model that correlates gene expression with hexose phosphate concentration.

Figure 1

Figure 2

A

B

Figure 3

Figure 4

Figure 5

A

B

Figure 6

TABLE 1. Quality parameters of the LCMS method

Metabolite	Molecular weight	Retention time (min)	MRM transition	Linearity range (μ M)	Hepatocytes		HepG2	
					LOD (μ M)	LOQ (μ M)	LOD (μ M)	LOQ (μ M)
HexoseP (G6P, F6P)	260	3.4	259/97	2-30	0.081	0.270	0.047	0.157
PentoseP (R5P, X5P)	230	3.7	229/97	1-16	0.053	0.177	0.051	0.170
2-DOG-6-P	244	3.6	243/97	8-30	0.144	0.481	0.146	0.487
1-DOX-5-P	214	4.1	213/97	9-20	0.311	1.038	0.246	0.821

LOD, Limit of detection; LOQ, limit of quantification; G6P, glucose-6-phosphate; F6P, fructose-6-phosphate; R5P, ribose-5-phosphate; X5P, xylulose-5-phosphate; 2-DOG-6-P, 2-deoxyglucose-6-phosphate; 1-DOX-5-P, 1-deoxyxylulose-5-phosphate

TABLE 2. Correlation coefficients (r^2) shown by the regression models

Incubation time	Correlation coefficient (r^2)			
	FAS		L-PK	
	6 h	18 h	6 h	18 h
M(H&P) ^a	0.998	0.960	0.979	0.987
M(H) ^b	0.957	0.938	0.992	0.855

^a Linear regression model that correlates gene expression with the product of hexose phosphate by pentose phosphate concentrations. ^b Linear regression model that correlates gene expression with hexose phosphate concentration.