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The inhibitory effect of glucose on phosphoenolpyruvate carboxykinase gene expression in cultured hepatocytes is transcriptional and requires glucose metabolism

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Abstract Phosphoenolpyruvate carboxykinase (PEPCK) is the rate-limiting enzyme of gluconeogenesis in the liver. PEPCK gene expression is controlled at the transcriptional level and is mainly regulated by hormones that are involved in glucose homeostasis. In this study, we have investigated the role of glucose on PEPCK gene expression in cultured hepatocytes. We demonstrate that glucose counteracts the stimulatory effect of glucocorticoids and cAMP on PEPCK expression. Glucose must be metabolized through glucokinase to have its inhibitory effect. The effect of glucose is mainly transcriptional and the region responsible for glucose inhibition is localized in the first 490 bp of the promoter.

Key words: Phosphoenolpyruvate carboxykinase; Glucose; Liver; Transcription; Glucose-6-phosphate

1. Introduction

It is now established that glucose stimulates the transcription of several genes encoding enzymes belonging to the glycolytic and lipogenic pathway in the liver, adipose tissue and pancreatic β-cells. This is the case for fatty acid synthase (FAS) and acetyl-CoA carboxylase (ACC), two key enzymes of the lipogenic pathway, L-pyruvate kinase (L-PK) involved in hepatic glycolysis and the protein of unknown function Spot 14 (S14) [1,2]. Studies performed in cultured adipocytes, cultured hepatocytes and β-cell insulinomas have clearly demonstrated that glucose must be metabolized to activate the transcription of these genes [3–8]. In the liver, the presence of glucokinase, which is responsible for glucose phosphorylation into glucose-6-phosphate (Glu-6-P), is crucial. The identity of the signal metabolite involved in the glucose effect remains controversial. We proposed that Glu-6-P could be a good candidate acting as a signal for glucose responsive genes [3,7,8]. On the other hand, Doiron et al. favored the hypothesis of the involvement of xylulose-5-phosphate, an intermediate of the pentose phosphate pathway [9].

Conversely, glucose can inhibit the transcription of various genes. The most well-known example are the genes encoding the family of glucose-regulated proteins (GRP). These proteins are localized within the endoplasmic reticulum and the Golgi apparatus and their expression is induced by a variety of stresses such as calcium ionophores, reducing agents or glucose deprivation [10]. Two genes encoding metabolic enzymes, the ubiquitous glucose transporter and aspartate aminotransferase (one enzyme of the neoglucogenic pathway in the liver and glyceroneogenic pathway in the adipose tissue), were described as members of the GRP family because of their response to glucose and stress treatment [11,12].

Phosphoenolpyruvate carboxykinase (PEPCK; EC 4.1.1.32) is the rate-limiting enzyme of gluconeogenesis in the liver. PEPCK gene expression is controlled at the transcriptional level and is mainly regulated by hormones that are involved in glucose homeostasis. PEPCK gene transcription is induced by glucagon, glucocorticoids and inhibited by insulin [13,14]. Concurrently to the well-known inhibition by insulin, several papers have described the inhibition of PEPCK gene expression by glucose [15–17].

In H4IIE hepatoma cells which do not express glucokinase, PEPCK gene expression is insensitive to glucose. Adenovirus-mediated transfection of this cell line with a glucokinase expression vector confers glucose sensitivity to PEPCK gene expression [17]. This suggested that the glucose effect requires glucose metabolism.

In the present work, we have investigated the effect of glucose on PEPCK gene expression in primary cultures of hepatocytes. We demonstrate that glucose inhibits PEPCK expression only when it can be metabolized. The effect of glucose is essentially transcriptional and the region responsible for glucose inhibition is localized in the first 490 bp of the promoter.

2. Materials and methods

2.1. Animals

Animal studies were conducted according to the French Guidelines for the Care and Use of Experimental Animals. Female Wistar rats (200–300 g body weight) fed ad libitum or 13 day old suckling rats were used (Iffa-Credo, L'Arbresle, France). They were housed in plastic cages at a constant temperature (22°C) with light from 07:00 h to 19:00 h for at least 1 week before the experiments.

2.2. Isolation, culture and transfection of hepatocytes

Hepatocytes were isolated by the collagenase method [18]. Cell viability was assessed by the trypan blue exclusion test and was always higher than 85%. Hepatocytes were seeded at a density of 8×10^6 cells (in 100 mm Petri dishes for RNA extraction) or 2×10^6 cells (in 60 mm Petri dishes for transfection experiments) in medium M199 with

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Abbreviations: PEPCK, phosphoenolpyruvate carboxykinase; Bt_2cAMP, dibutyryl cyclic adenosine monophosphate; DRB, 5,6-dichlorobenzimide riboside; Glu-6-P, glucose-6-phosphate; FAS, fatty acid synthase; L-PK, L-pyruvate kinase; S14, Spot 14

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Earle salts (Life technologies, Paisley, UK) supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 0.1% (w/v) bovine serum albumin, 2% (v/v) Ultroser G (Life Technologies), 100 nM dexamethasone (Sigma, St. Louis, MO, USA), 1 nM insulin (Actrapid, Novo Nordisk, Copenhagen, Denmark), 100 nM triiodothyronine (Sigma). After a 4 h attachment period, hepatocytes were cultured for 16–18 h in the presence of 5 mM glucose, 100 nM dexamethasone and 100 μM dibutyryl cyclic adenosine monophosphate (Bt_2cAMP) in order to induce PEPCK gene expression. Then, the culture medium was removed and replaced by a fresh medium containing the same concentrations of dexamethasone and Bt_2cAMP and increasing concentrations of glucose (5–25 mM). Hepatocytes were cultured during 24 h and harvested for RNA extraction.

For the PEPCK mRNA stability experiments, hepatocytes were cultured overnight in the presence of 100 nM dexamethasone, 100 μM Bt_2cAMP and 5 mM glucose. Then, the medium was replaced by a fresh similar medium containing 100 μM of the transcription inhibitor 5,6-dichlorobenzimide riboside (DRB) (Sigma) and 5 or 25 mM glucose.

A *Xba*I/*Bgl*II fragment corresponding to the first 490 bp of the rat PEPCK promoter was subcloned into pBLCAT2 (pCK490-CAT) (American Type Culture Collection, Rockville, MD, USA) and was used for transfection experiments using *N*-(1-(2,3-dioleoyloxy) propyl)-*N,N,N*-trimethylammonium methyl-sulfate (DOTAP) liposomal transfection reagent (Roche Molecular Biochemicals). Typically, 2×10^6 hepatocytes in 60 mm dishes were transfected with 5 μg of pCK490-CAT and 30 μl of DOTAP (1 μg/μl). After 12–14 h, the medium containing the DNA/liposome was removed and replaced by a medium containing or not 100 nM dexamethasone, 100 μM Bt_2cAMP and 5 or 25 mM glucose. Cells were then cultured further for 48 h and harvested for chloramphenicol acetyltransferase (CAT) assay as previously described [19]. CAT activity is expressed relative to the total amount of proteins in cellular extracts.

2.3. Metabolite concentration assay

The intracellular concentration of Glu-6-P in cultured hepatocytes was measured by a previously described spectrophotometric assay [8]. The medium lactate concentration was assayed according to [20].

2.4. Isolation of total RNA and Northern blot hybridization

Total cellular RNAs were extracted from hepatocytes using the guanidine thiocyanate method [21] and prepared for Northern blot hybridization as previously described [22]. Labelling of each probe was performed by random priming (Rediprime labelling kit, Amersham). Autoradiograms of Northern blots were scanned and quantified using an image processor program. PEPCK cDNA was as previously described [22]. Each Northern blot was hybridized with a ribosomal 18S probe in order to verify that equivalent amounts of total RNA were loaded in each lane.

2.5. Statistical analysis

Results are expressed as means ± S.E.M. Statistical analysis was performed with Student's *t*-test for unpaired data. When quantified, PEPCK mRNA concentrations were normalized with respect to the 18S hybridization signal.

3. Results

Glucocorticoids and glucagon acting via cAMP are both strong activators of PEPCK gene expression in the liver [23,24]. In a first series of experiments, we have tested whether high glucose concentrations could inhibit glucocorticoids and cAMP-activated PEPCK gene expression in hepatocytes. We have thus cultured adult rat hepatocytes for 24 h with increasing concentrations of glucose (5–25 mM) in the presence of dexamethasone, a glucocorticoid analog (Fig. 1A), Bt_2cAMP (Fig. 1B) or the combination of Bt_2cAMP and dexamethasone (Fig. 1C). We observed that whatever the activator of PEPCK gene expression, glucose is highly effective in counteracting the stimulatory effect of hormones. The inhibitory effect of glucose is already visible at a 10 mM concentration and is increased at higher concentrations (Fig. 1). In all the following

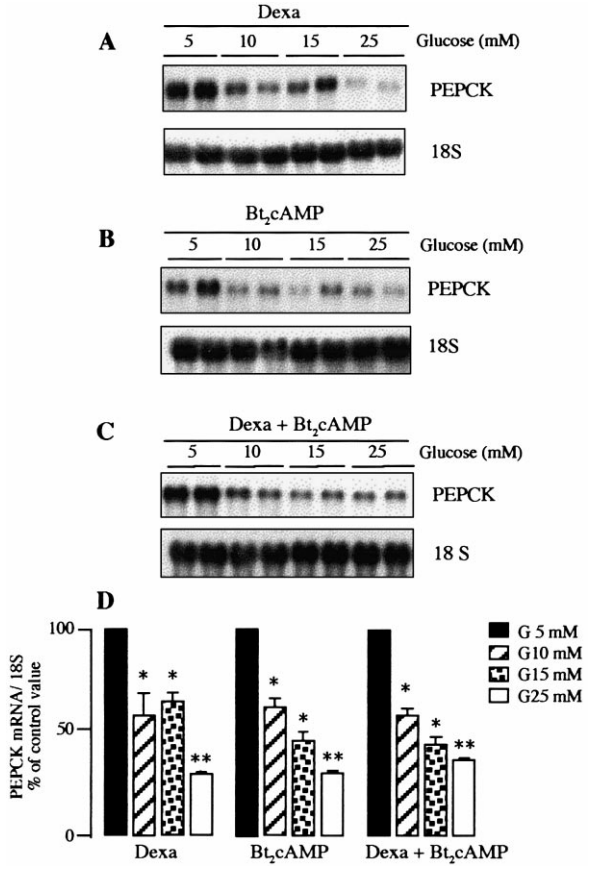

Fig. 1. Effect of increasing concentrations of glucose on glucocorticoid- and cAMP-activated PEPCK gene expression in cultured adult rat hepatocytes. Hepatocytes were cultured for 24 h in the presence of 5, 10, 15 or 20 mM glucose and 100 nM dexamethasone (dexa) (A), 100 μM Bt_2cAMP (B) or 100 nM dexamethasone plus 100 μM Bt_2cAMP (C). At the end of the culture, total RNAs were extracted and analyzed for the expression of PEPCK mRNA and 18S rRNA. A representative Northern blot as well as the quantification of blots obtained in three different experiments are shown. When quantified, the concentrations of mRNA are expressed as a ratio to the corresponding 18S signal and as a percentage of the value obtained in the presence of 5 mM glucose. *, **: Differences statistically significant for respectively $P<0.05$ and $P<0.01$ when compared to the value obtained in the presence of 5 mM glucose.

experiments, we have chosen to induce PEPCK gene expression overnight with dexamethasone and Bt_2cAMP in order to study the mechanism of glucose inhibition.

The inhibitory effect of glucose on PEPCK gene expression can be mediated by glucose itself or by one of its metabolites. We have then studied the effect of 3-O-methyl glucose (a glucose analog which is transported into the cell but not phosphorylated) on PEPCK gene expression. 3-O-methyl glucose at 10 or 25 mM has no inhibitory effect (Fig. 2), suggesting that glucose needs to be metabolized to achieve its effect. In addition, this shows that the glucose effect is not linked to an osmotic mechanism.

It has been clearly demonstrated that in cultured hepatocytes, the induction by glucose of glycolytic or lipogenic enzymes gene expression requires the presence of a highly effective glucokinase [6,7]. In the next experiment, we have compared the effect of glucose on PEPCK gene expression in fed adult rat hepatocytes and in 13 day old suckling rat hepatocytes which do not express glucokinase [25]. We have thus

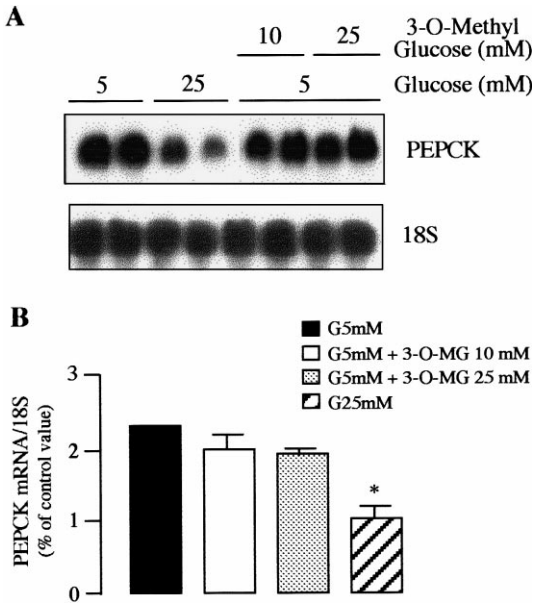

Fig. 2. Effect of 3-O-methyl glucose on PEPCK gene expression in cultured adult rat hepatocytes. Hepatocytes were cultured for 24 h in the presence of 100 nM dexamethasone, 100 μM Bt_2cAMP and in the presence of 25 mM glucose or 5 mM supplemented or not with 10 or 25 mM 3-O-methyl glucose. At the end of the culture, total RNAs were extracted and analyzed for the expression of PEPCK mRNA and 18S rRNA. A representative Northern blot as well as the quantification of blots obtained in three different experiments are shown. When quantified, the concentrations of mRNA are expressed as a ratio to the corresponding 18S signal and as a percentage of the value obtained in the presence of 5 mM glucose. Results are presented as the mean ± S.E.M. for three independent experiments. *, Difference statistically significant for $P < 0.05$ when compared to the value obtained in the presence of 5 mM glucose. ←

cultured hepatocytes in the presence of Bt_2cAMP and dexamethasone and increasing concentrations of glucose for 24 h.

The capacity of glucokinase to phosphorylate glucose was assessed by the measurement of Glu-6-P concentrations 1 h after the addition of the various glucose concentrations and by the measurement of lactate production in the medium after 24 h of culture. PEPCK mRNA expression was also measured after 24 h of culture. In suckling rat hepatocytes, Glu-6-P concentrations remain very low, even in the presence of a high glucose concentration in the medium (Fig. 3D). Similarly, lactate production in the medium is nearly undetectable, indicating that glucose metabolism through glucokinase is minimal in these hepatocytes (Fig. 3D). Interestingly, in suckling rat hepatocytes, glucose is totally ineffective in inhibiting PEPCK gene expression (Fig. 3C). Conversely, in adult rat hepatocytes, the inhibition of PEPCK gene expression by glucose (Fig. 3A) is concomitant with the presence of an effective glucokinase activity as shown by the increasing Glu-6-P concentrations and increasing lactate production in response to glucose (Fig. 3B). These data indicate that the presence of a

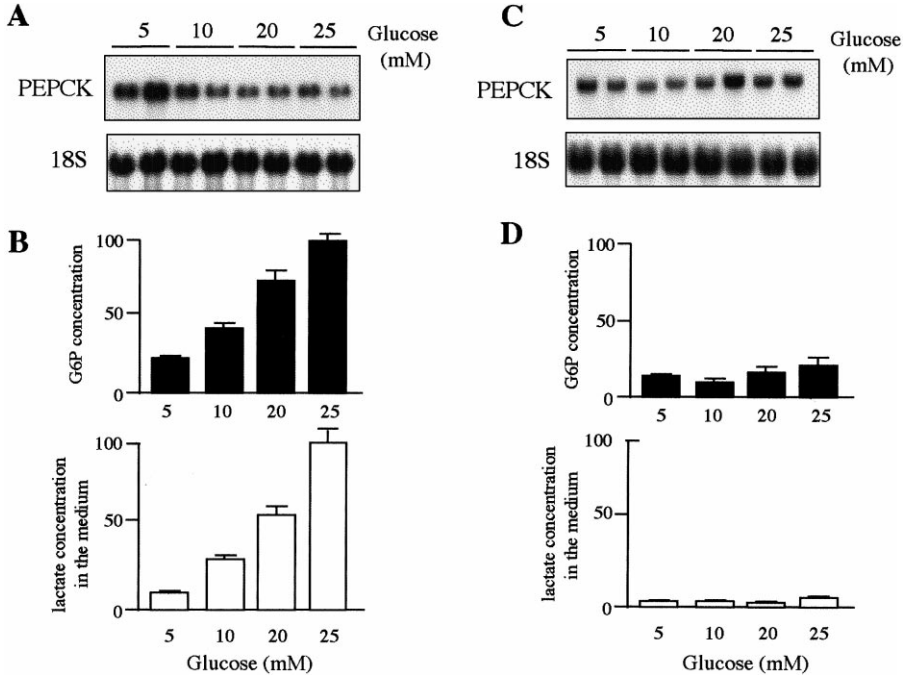

Fig. 3. Comparison of the effect of glucose on PEPCK gene expression in cultured adult and suckling rat hepatocytes. Determination of Glu-6-P and lactate concentrations. Adult or 15 day old suckling rat hepatocytes were cultured in the presence of 100 nM dexamethasone, 100 μM Bt_2cAMP and 5, 10, 20 or 25 mM glucose. Cells were harvested after 1 h for the determination of Glu-6-P concentrations or 24 h for total RNA extraction and lactate determination. Total RNAs were analyzed for the expression of PEPCK mRNA and 18S rRNA in adult (A) or suckling (C) rat hepatocytes. The Northern blot presented is representative of at least three independent experiments. Glu-6-P and lactate concentrations were determined in triplicate for each condition in adult (B) or suckling (D) rat hepatocytes. The results are expressed as percentage of the values obtained in the presence of 25 mM glucose in adult hepatocytes and as means ± S.E.M. for three independent cultures. Concentrations of Glu-6-P and lactate in the medium were respectively 2.2 ± 0.2 nmol/10^6 hepatocytes and 310 ± 22 μM/10^6 hepatocytes in the presence of 25 mM glucose in adult hepatocytes.

glucokinase activity is necessary for the inhibition of the PEPCK gene by glucose in hepatocytes.

We have next investigated the mechanisms by which glucose inhibits PEPCK gene expression in hepatocytes. At first, we have analyzed whether glucose affects the stability of PEPCK mRNA. Hepatocytes were cultured for 16 h in the presence of Bt_2cAMP and dexamethasone to induce a high expression of PEPCK. Then, the cells were incubated for 6 h in the presence of 100 μM DRB, an inhibitor of transcription, and in the presence of a normal glucose (5 mM) or a high glucose (25 mM) concentration. After blockade of the transcription process, the decrease of PEPCK mRNA was similar in the presence of 5 or 25 mM glucose (Fig. 4), indicating that the inhibitory effect of glucose on PEPCK gene expression does not involve a destabilization of PEPCK mRNA.

Finally, we have studied whether glucose acts at a transcriptional level to inhibit PEPCK gene expression. Adult rat hepatocytes were transfected by lipofection with the −490 bp promoter region of PEPCK linked to a CAT reporter gene. Then, the cells were cultured for 48 h in the absence or presence of Bt_2cAMP and dexamethasone and 5 or 25 mM glucose. Treatment of the hepatocytes with Bt_2cAMP and dexamethasone induces strongly the activity of the PEPCK promoter (Fig. 5). In the presence of 25 mM glucose, a 4-fold inhibition of PEPCK transcription was observed, indicating that glucose acts by modification of the transcription rate of the PEPCK gene and that the glucose response element is localized in the first 490 bp of the promoter.

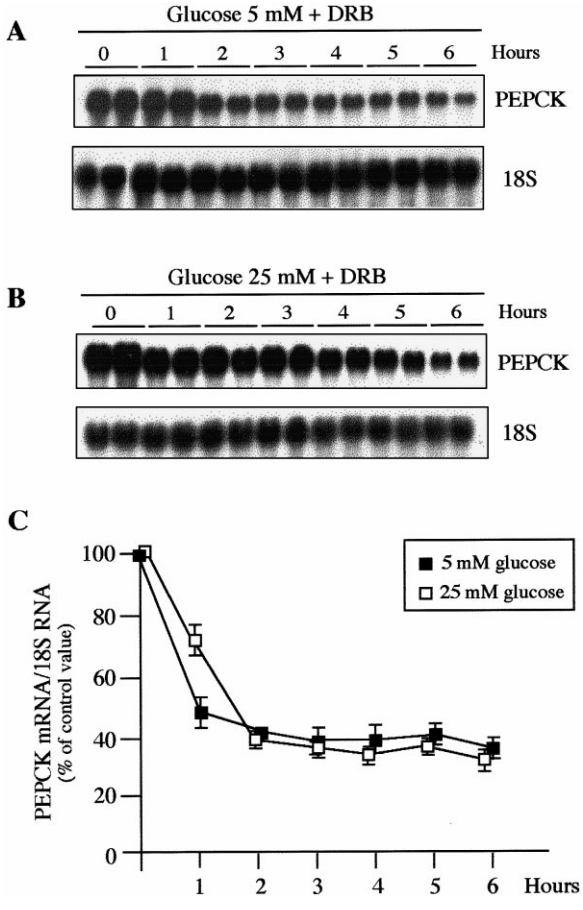

Fig. 4. Effect of glucose on PEPCK mRNA stability in cultured adult rat hepatocytes. Hepatocytes were cultured overnight (18 h) in the presence of 100 nM dexamethasone, 100 μM Bt_2cAMP and 5 mM glucose. Then, cells were incubated with a fresh similar medium containing 5 or 25 mM glucose and 100 μM DRB for 1, 2, 3, 4, 5 or 6 h. At the end of the culture, total RNAs were extracted and analyzed for the expression of PEPCK mRNA and 18S rRNA. A representative Northern blot as well as the quantification of blots obtained in two different experiments are shown. When quantified, the concentrations of mRNA are expressed as a ratio to the corresponding 18S signal and as a percentage of the value obtained after the overnight culture with dexamethasone and Bt_2cAMP.

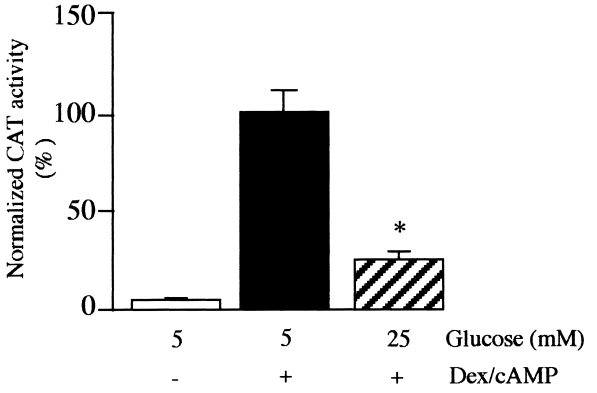

Fig. 5. Effect of glucose on the hormone-activated PEPCK gene promoter activity in cultured rat hepatocytes. Hepatocytes were transfected by lipofection with 5 μg of pCK490-CAT and 30 μl DOTAP (1 μg/μl) as described in Section 2. After 12–14 h, the medium containing the DNA/liposome was removed and replaced by a medium containing or not 100 nM dexamethasone, 100 μM Bt_2cAMP and 5 or 25 mM glucose. Cells were then cultured further for 48 h and harvested for the determination of CAT activity. CAT activity is expressed relative to the total amount of proteins in cellular extracts. Results are expressed as a percentage of the activity measured in the presence of 5 mM glucose, dexamethasone and Bt_2cAMP. Results are presented as the mean ± S.E.M. for three independent experiments. *, Difference statistically significant for $P < 0.05$ when compared to the value obtained in the presence of 5 mM glucose, dexamethasone and Bt_2cAMP.

4. Discussion

The liver plays a major role in glucose homeostasis. When the plasma glucose concentration is high, the liver synthesizes glycogen and lipids, the latter being ultimately stored in adipose tissue as triglycerides. When the plasma glucose concentration decreases, the liver produces glucose by the glycogenolytic and the gluconeogenic pathways. Initially, it was assumed that insulin secreted by the β-cells in response to carbohydrates regulates at both the post-translational and transcriptional level the activity of these pathways. More recently, studies performed in cultured cells have pointed out a role of glucose in the long term regulation of the glycolytic and lipogenic pathways. Indeed, it is now clear that both insulin and glucose are required to induce the expression of genes encoding lipogenic enzymes, ACC and FAS, the lipogenic related protein S14 and the glycolytic enzyme L-PK [1,2]. In addition, for this class of genes, the transcriptional effect of glucose requires prior metabolism through glucokinase [6,7]. The roles of insulin and glucose are different. Insulin acts by promoting the transcriptional activity of the transcription factor SREBP-1c. SREBP-1c in turn is sufficient to activate the expression of glucokinase [26] and is necessary

but not sufficient to induce FAS, ACC, L-PK and S14 transcription [27]. A high rate of glucose metabolism then potentiates the effect of SREBP-1c on the promoter of FAS, ACC, L-PK and S14 by a still unknown mechanism [26].

Concerning PEPCK expression, insulin alone, in the absence of a high glucose concentration, has an inhibitory effect [15]. We (present study) and others have shown that the inhibitory effect of glucose does not require the immediate presence of insulin. Taking advantage of a physiological model, hepatocytes from suckling rats which do not express glucokinase, we demonstrate that in primary hepatocytes, glucose must be metabolized to have its transcriptional inhibitory effect. This is in agreement with experiments showing that overexpression of glucokinase in the livers of diabetic transgenic mice is accompanied by a decrease in the mRNA encoding PEPCK and tyrosine aminotransferase [28] and with experiments showing that in H4IIE hepatoma cells which express only hexokinase I, the inhibitory effect of glucose requires an adenoviral-mediated overexpression of glucokinase [17]. Since glucokinase expression is under the strict dependency of insulin, it implies that the glucose effect on PEPCK is indirectly insulin dependent. This has probably previously been obscured by the fact that in adult rat hepatocytes cultured in the absence of insulin, a strong activity of glucose phosphorylation (see for instance Fig. 3) is still present due to the long half-life of the glucokinase protein (30 h) [29].

The nature of the glucose signal metabolite is unknown. In previous experiments in adipose tissue, we have shown that lactate and pyruvate were unable to mimic the repressive effect of glucose on PEPCK gene expression [30]. This suggests that the metabolite belongs to the upper part of the glycolytic pathway or to the pentose phosphate pathway. In a recent study performed in rats perfused with xylitol, it has been suggested that xylulose-5-phosphate might be the metabolite involved in PEPCK gene inhibition by glucose [31]. Xylulose-5-phosphate was also proposed by Doiron et al. as the metabolite involved in the activation of the L-PK gene [9]. However, in cultured hepatocytes, a high glucose concentration which induces FAS, ACC, L-PK and S14 and represses PEPCK expression does not induce a detectable increase in xylulose-5-phosphate [8]. Interestingly enough, in HL1C hepatoma cell lines, the glucose analog 2-deoxyglucose which is not readily metabolized beyond 2-deoxyglucose-6-phosphate is able to repress PEPCK expression, an argument rather in favor of Glu-6-P as the signal metabolite [17]. We have proposed from various experiments that for the activating effects of glucose, Glu-6-P could indeed be the activating factor [3,8]. Further experiments will be necessary to determine the exact nature of the glucose metabolite involved in stimulatory and inhibitory transcriptional effects.

The glucose effect on PEPCK gene expression in hepatocytes clearly involves a transcriptional mechanism. First, glucose has no effect on PEPCK mRNA stability (Fig. 4). Second, the activity of a −490 bp fragment of the PEPCK promoter linked to a reporter gene is strongly inhibited by glucose. This extends previous observations obtained in H4IIE hepatoma cell lines showing a repressive effect of glucose on a −2 kb fragment of the PEPCK promoter [17]. Interestingly enough, the −490 bp region of the promoter contains liver-specific elements and the elements responsible for the hormonal regulation of PEPCK expression in the liver. The cAMP response element is located between −90 and −83 bp on the PEPCK promoter [32] and the glucocorticoid regulatory unit which contains two glucocorticoid response elements [33] and an insulin response element is located from −415 to −440 bp of the promoter [34]. Glucose is able to repress both glucorticoid and cAMP-induced PEPCK expression. This might indicate that the target of glucose is a protein which integrates these different signals. Recently, the transcriptional co-activator CREB binding protein (CBP) was proposed as the coordinator of the factors which control transcription of the PEPCK gene [35]. An attractive hypothesis would be that CBP is indeed the glucose target.

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