Regulation of mammalian pyruvate dehydrogenase α subunit gene expression by glucose in HepG2 cells

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We report the effect of glucose on the expression of the gene encoding the pyruvate dehydrogenase (E1) α subunit (E1 α) in human hepatoma (HepG2) cells. Total pyruvate dehydrogenase complex activity as well as the levels of protein and mRNA of the E1 α subunit were significantly increased in HepG2 cells cultured in medium containing 16.7 mM glucose compared with 1.0 mM glucose for a period of 4 weeks. The level of E1 α mRNA was elevated approx. 2-fold in HepG2 cells cultured for 24 h in medium containing 16.7 mM glucose compared with 1 mM glucose. This effect was specific to glucose and independent of insulin. Nuclear run-on assays and promoter analysis indicate that the glucose-induced increases in the levels of E1 α mRNA in HepG2 cells are due to increased transcription of the human E1 α

(*PDHAI*) gene. Mutational analysis of the E1 α promoter region has identified two regions, from –78 to –73 bp (CCCCTG) and from –8 to –3 bp (GCGGTG), that are responsible for the effect of glucose on promoter activity; the former exhibits a larger effect. These two sequences represent new variations of the carbohydrate-response element that has been identified in other genes. The stimulation of E1 α promoter activity by glucose was abolished by okadaic acid at 100 nM but not at 5 nM, suggesting that glucose-mediated regulation of pyruvate dehydrogenase complex E1 α gene transcription involves a phosphorylation/dephosphorylation mechanism, possibly involving protein phosphatase-1.

INTRODUCTION

Recently, a number of genes encoding several key enzymes involved in the control of glucose and fatty acid metabolism have been shown to be regulated by glucose at the transcriptional level. These proteins include the glucose transporter GLUT2, L-pyruvate kinase, acetyl-CoA carboxylase, the S14 gene product and fatty acid synthase [1–4]. A carbohydrate-response element (ChoRE) has been identified in these promoters [5–9]. Although extensive studies have been conducted on the L-pyruvate kinase and S14 genes, little else is known about the glucose-mediated regulation of the genes involved in the glucose oxidation pathway. The pyruvate dehydrogenase complex (PDC) catalyses a rate-limiting step in glucose oxidation [10]. It irreversibly converts pyruvate into acetyl-CoA, which is either oxidized further via the tricarboxylic acid cycle for energy generation or used in the synthesis of fatty acids and cholesterol in lipogenic tissues.

Despite the well established mechanism of regulation of PDC enzymic activity [10,11], the regulatory mechanism for PDC gene expression has not been well characterized. Although the total PDC activity in animal tissue is relatively constant under normal conditions [12], suggesting that the PDC genes may be expressed at constant levels, there are reports of changes in total PDC activity on a long-term basis. For example, high-carbohydrate diets increase the total PDC activity by approx. 2-fold in rat liver and adipose tissues [13–17]. Also, the levels of PDC proteins and their respective mRNAs in rat adipose tissue are increased at the transition from suckling to weaning, due to the change of diet from milk (high fat) to a high-carbohydrate diet [18]. Moreover,

the E1 α subunit (E1 α) mRNA level is increased several-fold by glucose in cultured rat pancreatic islets [19], suggesting that the expression of PDC genes may be under nutritional and/or hormonal control.

In the present paper, we report the glucose-mediated regulation of total PDC activity and PDC E1 α gene expression in HepG2 cells. The results show that the effect of glucose on PDC E1 α gene expression is at the transcription level, and that two variant forms of the ChoRE sequence in the proximal promoter region of the human E1 α gene are responsible for this effect.

EXPERIMENTAL

Cell culture

Human hepatoma (HepG2) cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Life Technologies) containing glucose at the concentrations indicated elsewhere and supplemented with 10% (v/v) heat-inactivated fetal bovine serum, 100 units/ml penicillin and 100 μ g/ml streptomycin. At 50% confluence, cells were washed with PBS and cultured in DMEM containing 1 mM glucose with or without serum for 20 h, and then were treated with several nutritional compounds (as indicated in the Figure legends) for another 20–24 h.

Total PDC activity assays

Total PDC activity was measured essentially as previously described [20]. Briefly, HepG2 cells maintained for approx. 4

Abbreviations used: ChoRE, carbohydrate-response element; PDC, pyruvate dehydrogenase complex; E1, pyruvate dehydrogenase component of PDC; E1 α , α subunit of E1; E3, dihydrolipoamide dehydrogenase; DMEM, Dulbecco's modified Eagle's medium; CAT, chloramphenicol acetyltransferase; AP2, activator protein 2; PP-1, protein phosphatase-1; PP-2A, protein phosphatase-2A; USF, upstream stimulatory factor.

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weeks in complete DMEM (with 10 % serum) containing either 1.0 mM or 16.7 mM glucose were homogenized in 300 μ l of 50 mM Hepes (pH 7.5), 0.2 mM KCl, 3 mM EDTA, 5 mM dithiothreitol, 0.5 μ g/ml leupeptin, 0.5 mM PMSF and 0.25 % (v/v) Triton X-100. The homogenates were centrifuged for 10 min at 500 *g* and the supernatants were saved. Protein concentrations of the supernatants were measured using the Bradford assay (Bio-Rad) according to the manufacturer's instructions. Before measuring the PDC activity, the supernatants were twice frozen and thawed. Samples were activated in 10 mM MgCl_2 /1 mM CaCl_2 buffer containing dichloroacetate (5.6 mM) and purified E1 phosphatase (0.7 unit/ml; kindly provided by Dr. Thomas Roche, Kansas State University, Manhattan, KS, U.S.A.) at 37 °C for 15 min. The activated crude extracts were subsequently assayed by measuring the decarboxylation of [^{14}C] pyruvate into $^{14}\text{CO}_2$. The reactions were stopped by adding trichloroacetic acid. The $^{14}\text{CO}_2$ was quantified using a scintillation counter. One m-unit of PDC activity is defined as the production of 1 nmol of CO_2 /min at 37 °C. Fold changes are shown in Figure 1.

Western blot analysis

Cell extracts prepared as described above were used for an immunoblotting assay, performed essentially as previously described [21], except that the incubation with antibodies was at room temperature. After incubation with anti-PDC antibodies (rabbit anti-bovine) [20], the blot was washed with PBS/0.1 % Tween-20 and incubated with horseradish peroxidase-conjugated goat anti-rabbit antibody. PDC protein was detected using a chemiluminescence Western blotting kit (DuPont, NEN) according to the manufacturer's instructions and quantified using a GS700 laser scanner (Bio-Rad) and Molecular Analysis software (Bio-Rad).

RNA analysis

Total RNA was isolated by the standard guanidinium isothiocyanate method [22]. Samples of 10–20 μ g of RNA denatured in glyoxal buffer in 50 °C for 1 h were subjected to electrophoresis and transferred to a nylon membrane. The membrane was incubated with ^{32}P -labelled E1 α or E1 β cDNA probes in Church buffer [23] at 68 °C overnight, washed twice with $2 \times \text{SSC}/2 \%$ SDS ($1 \times \text{SSC}$ is 0.15 M NaCl/0.015 M sodium citrate) at 68 °C for 5 min and three times with $0.2 \times \text{SSC}/0.2 \%$ SDS at 68 °C for 30 min, and then exposed to Kodak X-AR films at $-80 \text{ }^\circ\text{C}$ for 24–48 h. β -Actin cDNA or a 50 bp oligonucleotide probe specific to 18 S rRNA was used as internal control. The autoradiogram was analysed using a densitometer (Bio-Rad), and the E1 α or E1 β mRNA level was normalized to that of the β -actin or 18 S rRNA transcript.

Nuclear run-on analysis

Nuclei from HepG2 cells treated with 16.7 mM or 1 mM glucose in serum-free medium for 24 h were isolated as described [24]. A transcription elongation reaction was carried out in 100 μ l containing 32 % glycerol, 100 mM KCl, 50 mM Hepes (pH 7.4), 5 mM magnesium acetate, 4 mM dithiothreitol, 0.1 mM EDTA, 4 units of RNasin, 1 mM each of CTP, GTP and ATP, and 0.5 mCi of [α - ^{32}P]UTP at 34 °C for 45 min. The reaction was terminated by the addition of 1 mM MgCl_2 and 10000 units of DNase I (RNase-free) at 37 °C for 15 min, then incubated with 300 μ g/ml proteinase K in the presence of 0.1 % SDS at 37 °C for 30 min. ^{32}P -labelled total nuclear RNA was then extracted and used for hybridization. Samples of 5 μ g of plasmid DNA

containing E1 α or β -actin cDNA or pBluescript vector DNA were denatured in 0.3 M NaOH for 10 min at room temperature and neutralized in $6 \times \text{SSC}$, and then dot-blotted on to Gene-Screen membranes (DuPont). Equal numbers of counts of the ^{32}P -labelled nascent RNAs [$(1-3) \times 10^7$ c.p.m./ml] were incubated with the membranes in Church buffer [23] at 68 °C for 2 days. Membranes were washed four times in $2 \times \text{SSC}$ for 5 min each at room temperature, incubated in $2 \times \text{SSC}$ containing RNase A (10 μ g/ml) at 37 °C for 30 min, and then washed twice for 15 min each in $0.2 \times \text{SSC}/1 \%$ SDS at 65 °C and exposed to Kodak X-AR films for 1–3 days. The autoradiogram was quantified using a GS700 laser scanning densitometer (Bio-Rad).

Construction of plasmids and site-directed mutagenesis

The 5.8 kb (-5.8 kb to $+33$ bp; J. Tan and M. S. Patel, unpublished work) or the 796 bp (-763 to $+33$ bp) [25] human E1 α promoter was inserted upstream of the chloramphenicol acetyltransferase (CAT) structure gene in pBluescript (Stratagene, but the β -galactosidase gene in the plasmid was deleted). Deletion constructs were generated by using restriction enzyme digestion at *AccI*, *ApaI* and *StuI* sites of the -763 bp promoter. Mutations at Sp1, activator protein 2 (AP2) and ChoRE sites were generated by using either the MORPH site-specific mutagenesis kit (5 Prime $\rightarrow$ 3 Prime) or the PCR method (Stratagene). The sequence resulting from each mutation was verified by DNA sequencing. Unless otherwise indicated, all promoter constructs contained the $+33$ bp region downstream of the transcription start site.

Transient CAT expression assay

HepG2 cells were maintained in DMEM containing 10 % (v/v) heat-inactivated fetal bovine serum, 10 mM glucose and 2 nM insulin. At 50 % confluence, cells were transiently transfected with 8 μ g of the testing plasmid and 3 μ g of pRSV- β -Gal by the calcium phosphate precipitation method [26]. After 10 h of transfection, cells were washed with PBS and then cultured in glucose-free DMEM supplemented with either 10 mM lactate or 16.7 mM glucose with or without okadaic acid. After additional incubation for 24 h, cells were harvested and CAT activity was measured by the phase-extraction method [27]. CAT activities were normalized against β -galactosidase activities, to minimize the variation caused by transfection efficiency.

Statistical analysis

Experimental data are expressed as means $\pm$ S.D. The statistical significance of differences between control and experimental treatments was assessed by Student's unpaired *t* test.

RESULTS

Glucose increases the human PDC E1 α mRNA level in HepG2 cells

Since several genes (e.g. those encoding L-pyruvate kinase and acetyl-CoA carboxylase) have been shown to be regulated by glucose in pancreatic β cells and liver cells [28], we investigated both the chronic and acute effects of glucose on human E1 α gene expression in HepG2 cells, as well as the effect of serum in the culture medium. To investigate the effects of the chronic exposure of human HepG2 cells to high glucose concentrations on PDC activity and the levels of E1 α protein and mRNA, the cells were cultured in medium containing 10 % (v/v) fetal bovine serum and either 1 or 16.7 mM glucose for 4 weeks. Total PDC activity and the relative levels of E1 α protein and mRNA were measured.

Figure 1 Chronic effects of glucose on total PDC activity and levels of PDC E1 α protein and mRNA in HepG2 cells

HepG2 cells were propagated and maintained for 4 weeks in DMEM supplemented with 10% (v/v) fetal bovine serum containing 1 mM or 16.7 mM glucose. Samples of 30 μ g and 25 μ g of total cellular extracts were used for the total PDC activity and E1 α protein immunoblotting assays respectively, and 10 μ g of total RNA was used for Northern blot analysis. (A) Total PDC activity (m-units/mg of cellular protein) in cells cultured in 1 mM and 16.7 mM glucose. (B), (D) Relative levels of E1 α protein in cells cultured in 1 mM and 16.7 mM glucose (B), and immunoblot results (D). (C), (E) Relative levels of E1 α mRNA in cells cultured in 1 mM and 16.7 mM glucose (C), and Northern hybridization results (E). The results obtained with cells cultured in 1 mM glucose were arbitrarily assigned a value of 1.0. The density of the E1 α mRNA band was normalized to that of 18 S rRNA in (C). Values represent means $\pm$ S.D. of at least three separate experiments; * P < 0.05 compared with control (1 mM glucose).

As shown in Figure 1, total PDC activity in 16.7 mM-glucose-cultured cells was approx. 2-fold higher than that of cells cultured in 1 mM glucose (Figure 1A). The increase in total PDC activity was reflected by an approx. 2-fold increase in E1 α protein in 16.7 mM-glucose-cultured cells compared with 1 mM-glucose-cultured cells (Figures 1B and 1D). Northern blot analysis was performed to see whether this change was reflected at the E1 α mRNA level. As depicted in Figures 1(C) and 1(E), the level of E1 α mRNA was increased by approx. 1.5-fold in 16.7 mM-glucose-cultured cells compared with that in 1 mM-glucose-cultured cells.

In another set of experiments to investigate the acute effects of high glucose concentrations on the level of E1 α mRNA, HepG2 cells routinely cultured in 10 mM glucose containing 10% fetal bovine serum were used. In order to observe the stimulatory effect of glucose, cells at 50% confluence were first cultured in medium containing 1 mM glucose with or without serum for 24 h, and were then cultured in medium containing 16.7 mM glucose with or without serum for 24 h. Total RNA was isolated and analysed by Northern blot assays. As shown in Figure 2, lowering the glucose concentration to 1 mM in the presence of serum decreased the E1 α mRNA level by about 40% compared with cells grown in 10 mM glucose/10% serum. Addition of 16.7 mM glucose to the cells pretreated with 1 mM glucose elevated the E1 α mRNA level more than 2-fold with or without

Figure 2 Acute effect of glucose on the level of E1 α mRNA in HepG2 cells

(A), (C) Northern hybridization analysis of E1 α gene expression. HepG2 cells were maintained in 10 mM glucose (Glc) in DMEM supplemented with 10% (v/v) fetal bovine serum (lanes 1, 2, 12 and 13). At 50% confluence, cells were shifted to 1 mM glucose medium with (lane 9) or without (lanes 3 and 4) 10% serum for 24 h. Cells were then washed, and maintained in the medium containing 16.7 mM glucose with (lanes 5, 6 and 10) or without (lanes 7, 8 and 11) 10% serum for another 24 h. Samples of 20 μ g of total RNA were subjected to Northern blot analysis for the level of E1 α mRNA; the β -actin mRNA level was used as an internal standard. (B), (D) The relative levels of E1 α mRNA in (A) and (C) were quantified by densitometer scanning. The levels of E1 α mRNA were normalized to that of β -actin. The levels of E1 α mRNA in 1 mM glucose culture were arbitrarily given a value of 1.0. Values represent means $\pm$ S.D. of three separate experiments; * P < 0.05 compared with control (1 mM glucose).

the presence of serum, suggesting that serum does not affect the E1 α mRNA level and that glucose is the primary cause of the elevation in the E1 α mRNA level. Therefore in subsequent experiments serum-free medium was used.

The dose-dependent effects of glucose on E1 α and E1 β mRNA levels in HepG2 cells were then examined; for comparison, the level of glucokinase mRNA was also examined. As shown in Figure 3, both E1 α and E1 β mRNA levels were elevated more than 2-fold in the presence of 5.5 mM glucose compared with those observed at 1 mM glucose, and these elevated levels were maintained even at 30 mM glucose. Therefore 5.5 mM glucose is fully effective in increasing human PDC E1 α and E1 β mRNA levels in HepG2 cells. The level of glucokinase mRNA was increased about 10-fold by 5.5 mM glucose, and was further increased another 2-fold by 30 mM glucose, indicating that the glucose effect on PDC gene expression is not as dramatic as that on glucokinase gene expression. The fold increase in glucokinase gene expression caused by glucose under our experimental conditions is in a range similar to that reported previously for the S14 and pyruvate kinase genes [1–4], indicating that HepG2 cells are fully capable of responding to a glucose stimulus. There is, however, a leftward shift in sensitivity with regard to the concentration of glucose required for an effect in HepG2 cells compared with primary hepatocytes. A similar leftward shift in glucose sensitivity has been observed for other tumour cell lines, and may reflect a change in the expression of glucose transporters in these cells (see the Discussion section). It should be noted that

Figure 3 Dose-dependence of the effect of glucose on the levels of PDC E1 α and E1 β mRNAs in HepG2 cells

(A) Autoradiogram of the Northern blot analysis of human PDC E1, E3 and glucokinase (GK) gene expression. HepG2 cells grown in 10 mM glucose medium were shifted to 1 mM glucose (Glc) serum-free medium for 24 h (lanes 1 and 2); they were then cultured with 5.5 mM glucose (lanes 3 and 4) or 30 mM glucose (lanes 5 and 6) serum-free medium for another 24 h. Samples of 20 μ g of total RNA were analysed. (B) The hybridization bands in (A) were quantified and the ratio relative to β -actin mRNA was taken as the arbitrary unit for the level of mRNA. The relative level of each specific mRNA in cells cultured in 1 mM glucose was arbitrarily assigned a value of 1.0. Values represent means $\pm$ S.D. of three separate experiments; * P < 0.01 compared with control (1 mM glucose).

E3 (dihydrolipoamide dehydrogenase) mRNA was also increased upon glucose treatment (Figure 3).

In order to examine the specificity of the glucose effect on E1 α gene expression, pyruvate, galactose or fructose (16.7 mM) was added to the HepG2 cells precultured in the medium containing 1 mM glucose. Total RNA was isolated and analysed by Northern blot assay (Figure 4). None of pyruvate, galactose and fructose increased the actual level of E1 α mRNA. It should be noted that, upon addition of galactose and fructose, the level of β -actin mRNA was dramatically increased (Figure 4A), resulting in an apparent decrease in the relative abundance of E1 α mRNA in cells treated with these two hexoses (Figure 4B). The presence of insulin in the medium also had no effect on the level of E1 α mRNA (results for insulin alone not shown). The lack of effect of insulin may be due to the dominant expression of GLUT1 in human liver tumour cells.

Regulation of E1 α gene expression by glucose is at the transcriptional level

In order to determine whether the increase in E1 α mRNA levels induced by glucose is due to an increase in the transcription rate or in mRNA stability, nuclear run-on analysis was performed. Nuclei from HepG2 cells cultured in 1 mM or 16.7 mM glucose were isolated and incubated with [32 P]ATP. The labelled nascent nuclear RNAs were then isolated and hybridized to the E1 α cDNA and β -actin cDNA probes immobilized on a nylon

Figure 4 Effects of insulin, pyruvate, galactose and fructose on the level of E1 α mRNA in HepG2 cells

(A) Autoradiogram of Northern hybridization analysis. HepG2 cells pre-cultured in 1 mM glucose-containing serum-free medium for 24 h (lanes 1 and 2) were cultured in serum-free medium containing 16.7 mM of the following compounds: fructose (Fru), lanes 3 and 4; pyruvate (Pyr), lanes 5 and 6; glucose (Glc), lanes 7 and 8; galactose (Gal), lanes 9–11; glucose plus 100 nM insulin (Ins), lanes 12 and 13. Samples of 20 μ g of total RNA were subjected to Northern blot analysis. (B) The hybridization bands in (A) were quantified. The ratios of the E1 α mRNA level to the β -actin mRNA level are compared. The relative level of E1 α mRNA in cells cultured in 1 mM glucose was arbitrarily assigned a value of 1.0. Values represent means $\pm$ S.D. of three separate experiments; * P < 0.01 compared with control (1 mM glucose). Abbreviation: CHO, carbohydrate.

membrane. The hybridization signal therefore represents the rate of transcription. The level of newly synthesized E1 α mRNA was about 2-fold higher in 16.7 mM-glucose-cultured cells than in 1 mM-glucose-cultured cells (Figure 5). The rate of β -actin mRNA synthesis did not change significantly. The pBluescript plasmid (without insert) was used for an indication of the background hybridization.

E1 α promoter activity is regulated by glucose

We further investigated the effect of glucose on the promoter activity of the human E1 α (*PDHA1*) gene in HepG2 cells. Different portions of the E1 α promoter region (with the +33 bp region from the transcription start site) were inserted upstream of a CAT reporter gene and transiently transfected into HepG2 cells. In the next series of experiments, we used 10 mM lactate in the medium (as a control) to provide more oxidizable substrate instead of 1 mM glucose, for two reasons: (i) in a preliminary study, we observed that expression of CAT activity in transfected HepG2 cells grown in the presence of 10 mM lactate was similar to that seen in cells cultured in medium containing 1 mM glucose (results not shown); and (ii) in order to compare our results of glucose-mediated CAT expression with findings previously reported for other genes [2,4]. After 10 h of transfection, cells were cultured in medium containing either 10 mM lactate (control) or 16.7 mM glucose for 24 h and then harvested. CAT activities in the supernatants of the cellular extracts were measured and normalized to the co-transfected β -galactosidase activity. All E1 α promoter constructs longer than the –100 bp region showed

Figure 5 Nuclear run-on analysis of the effect of glucose on PDC E1 α gene expression in HepG2 cells

(A) Nuclei were isolated from HepG2 cells cultured in serum-free medium containing either 1 mM or 16.7 mM glucose (Glc) for 24 h. 32 P-labelled nascent RNAs were isolated and equal amounts (2×10^7 c.p.m./ml) of each labelled nascent RNA were used to hybridize to the nylon membrane strip dotted with 5 μ g of E1 α cDNA, pBluescript (pBS) vector plasmid DNA or β -actin cDNA. pBS DNA was used as a control for non-specific hybridization, and β -actin cDNA was used as an internal standard. (B) The hybridization bands in (A) were quantified and the amount relative to β -actin mRNA was used as an arbitrary unit for the level of E1 mRNA. The relative level of each specific mRNA in cells cultured in 1 mM glucose was arbitrarily assigned a value of 1.0. Values represent means $\pm$ S.D. of three separate experiments. The relative PDC E1 α mRNA level of 16.7 mM-glucose-treated cells was 1.95 ± 0.25 ($P < 0.05$).

Figure 6 Glucose-responsiveness of a series of deletion constructs of the human E1 α gene promoter

Portions of 8 μ g of each construct were transiently transfected into HepG2 cells, along with 3 μ g of pRSV- β -Gal. Transfected cells were then cultured in medium containing either 10 mM lactate (Lac; solid bars) or 16.7 mM glucose (Glc; hatched bars) for 36 h. The expression of CAT is normalized to that of β -galactosidase, and fold changes upon glucose treatment are shown. p-763 is the $-763/+33$ bp E1 α promoter/CAT construct. In p-763/Intron, about 5 kb of the human PDC E1 α first intron was inserted at the 3' end of CAT gene in p-763. p-493, p-217, p-100 and p-50 are the $-493/+33$ bp, $-217/+33$ bp, $-100/+33$ bp and $-50/+33$ bp E1 α promoter/CAT constructs respectively. p-50/-10 is the $-50/-10$ bp E1 α promoter/CAT construct. The CAT activity (normalized to that of β -galactosidase) of p-763 in 10 mM lactate culture was arbitrarily assigned a value of 1.0. Values represent means $\pm$ S.D. of five to six separate experiments; $^*P < 0.01$ compared with control (10 mM lactate).

Figure 7 Glucose-responsiveness of mutant constructs of the -100 bp E1 α promoter

(A) Samples of 8 μ g of each construct (shown in B) were co-transfected with 3 μ g of pRSV- β -Gal into HepG2 cells under culture conditions as described in the legend to Figure 6 (Lac, 10 mM lactate; Glc, 16.7 mM glucose). The CAT expression (relative to that of β -galactosidase) of pWT-100 in 10 mM lactate culture was arbitrarily assigned a value of 1.0. Values represent means $\pm$ S.D. of five to six separate experiments; $^*P < 0.05$ and $^{**}P < 0.01$ compared with control (10 mM lactate). (B) Schematic representation of the mutation or deletion constructs in (A) and the sequence comparison among the ChoREs of the L-pyruvate kinase (L-PK) gene, the S14 gene and the human PDC E1 α promoter. pWT-100 is the $-100/+33$ bp E1 α promoter/CAT construct; pWT-83 is the $-83/+33$ bp E1 α promoter/CAT construct. pmSp1, pM-1, pM-2 and pmAP2 are mutant constructs of pWT-100. pWT-61 is the $-61/+33$ bp E1 α promoter/CAT construct; pM-3 and pM-4 are mutant constructs of pWT-61. p-50/-10 is the $-50/-10$ bp E1 α promoter/CAT construct (deletion of the -9 to $+33$ bp region).

2–3-fold stimulation of CAT activity in cells grown in 16.7 mM glucose compared with those grown in 10 mM lactate (Figure 6). Deletion of the promoter from -100 bp to -50 bp reduced the glucose stimulation to approx. 1.5-fold. Truncation of the $-10/+33$ bp region (p-50/-10 construct) resulted in the complete loss of glucose stimulation, indicating that the glucose-responsiveness of the E1 α promoter is sequence-specific. The *cis*-acting element may be located in the -100 to -50 bp and/or the -10 to $+33$ bp region of the E1 α promoter.

Localization of a possible ChoRE site in the E1 α promoter

In the -100 to -50 bp region of the E1 α promoter, an Sp1-binding site (-90 to -82 bp) and an AP2-binding site (-55 to -44 bp) were identified by *in vitro* footprinting in a previous study [25]. Both of these proteins have been shown to be involved in cAMP- or glucose-mediated regulation of acetyl-CoA carboxylase gene transcription [32,33]. Moreover, the sequences of the -82 to -58 bp and -20 to $+1$ bp regions of the E1 α promoter show some similarity to the ChoRE sequence in the promoter of the liver pyruvate kinase gene (Figure 7B). To investigate the possibility that at least one of these sites may be

Figure 8 Okadaic acid inhibits the glucose effect on human E1 α promoter activity

Samples of 8 μ g of the -763/+33 bp or the -5800/+33 bp E1 α promoter/CAT constructs, or of 5 μ g of pSV2CAT, were co-transfected with 3 μ g of pRSV- β -Gal into HepG2 cells. The CAT expression (relative to that of β -galactosidase) of each construct in 10 mM lactate culture was arbitrarily assigned a value of 1.0. Values represent means $\pm$ S.D. of five to six separate experiments; * P < 0.01 compared with control (10 mM lactate). The additions were: Glc, 16.7 mM glucose; Lac, 10 mM lactate; OA, okadaic acid; 6-DOG, 16.7 mM 6-deoxyglucose. Abbreviations: SV40, simian virus 40; pBS, pBluescript.

involved in the glucose regulation of the human PDC E1 α gene, mutation and deletion constructs of these sites were generated (Figure 7B) and transiently transfected into HepG2 cells.

As shown in Figure 7(A), neither mutation nor deletion of the Sp1 site in the -93 bp to -83 bp region (pmSp1 and pWT-83) affected the promoter activity or abolished the glucose effect. Mutation of the putative AP2 site (pmAP2) did not affect glucose responsiveness, although the mAP2 mutation did reduce promoter activity by about 50% in both glucose and lactate cultures. However, mutations (M-1 and M-2) spanning the -83 to -61 bp region reduced glucose stimulation to approx. 1.6-fold, compared with about 3-fold stimulation of the wild-type promoter. Consistent with this, deletion of the -83 to -61 region (pWT-61) decreased the glucose stimulation to about 1.4-fold with a higher promoter activity in both 16.7 mM glucose and the 10 mM lactate cell cultures. Further mutation of the -10 to -7 bp region (M-4) in the pWT-61 construct, which disrupted a ChoRE-like sequence (GCGGTG) from -8 to -3 bp, caused a complete loss of the glucose stimulation. A construct mutated in the -14 to -10 bp region (M-3) still showed a 1.4-fold stimulation by glucose, suggesting that the GCGGTG sequence (-8 to -3 bp) also contributes to the full glucose stimulation in the wild-type E1 α promoter.

Okadaic acid abolishes the glucose effect on E1 α promoter activity

A phosphorylation/dephosphorylation mechanism has been shown to be involved in the glucose regulation of the promoters of the S14 and acetyl-CoA carboxylase genes [34,35]. To see whether the glucose effect on the human PDC E1 α promoter also involves a phosphorylation/dephosphorylation mechanism, the effect of okadaic acid (a protein phosphatase inhibitor) on the glucose regulation of E1 α promoter activity was investigated. The -5.8 kb and -763 bp human PDC E1 α promoter/CAT

Figure 9 Effect of xylitol on CAT expression by the human E1 α promoter in HepG2 cells

Samples of 8 μ g of the -100 bp E1 α promoter/CAT construct and 3 μ g of the pRSV- β -Gal construct were co-transfected into HepG2 cells. Cells were cultured in the presence of 10 mM lactate (Lac) or 16.7 mM glucose (Glc), or in 10 mM lactate plus various concentrations of xylitol, in serum-free medium for 36 h. CAT activity was normalized to β -galactosidase activity. The normalized CAT activity in 10 mM lactate culture was arbitrarily assigned a value of 1.0. Values represent means $\pm$ S.D. of six separate experiments; * P < 0.05 and ** P < 0.01 compared with control (10 mM lactate).

constructs were transiently transfected into HepG2 cells. After 10 h of transfection, cells were cultured in medium containing either 10 mM lactate or 16.7 mM glucose with or without okadaic acid for an additional 24 h, and were then harvested. The CAT activity in each culture was measured. The effect of 6-deoxyglucose was also tested. As shown in Figure 8, 100 nM okadaic acid abolished the glucose stimulation of the promoter activities of both the -5.8 kb and -763 bp regions of the E1 α gene, but 5 nM okadaic acid had no effect. In contrast, 100 nM okadaic acid had no significant effect on the non-glucose-responsive simian virus 40 early promoter. Because protein phosphatase-2A (PP-2A) is fully inhibited by 5 nM okadaic acid, whereas protein phosphatase-1 (PP-1) is fully inhibited by 100 nM okadaic acid, *in vitro* and *in vivo* [36], PP-1 is more likely to be the phosphatase involved in the mechanism of glucose stimulation.

As expected, 16.7 mM 6-deoxyglucose did not stimulate the promoter activity of the E1 α gene, indicating that glucose has to be metabolized to generate the necessary signal for the glucose effect.

Xylitol does not stimulate PDC E1 α promoter activity

Xylitol, which is metabolized via the pentose phosphate pathway, has been shown to stimulate L-pyruvate kinase promoter activity through the ChoRE site at low concentrations (0.1–10 mM), with a maximal effect at 0.5 mM in primary rat liver cells [37]. We thus investigated the possible effect of xylitol on human PDC E1 α promoter activity. As shown in Figure 9, xylitol had no stimulatory effect up to 1 mM and showed an inhibitory effect from 2.5 to 10 mM on E1 α promoter activity, suggesting that xylitol (or its metabolite) is not the primary signal in the glucose regulation of E1 α gene transcription in HepG2 cells.

DISCUSSION

Regulation by nutrients of the expression of genes encoding several key regulatory enzymes in their metabolic pathways has been well documented [1,2,4]. However, the regulation of PDC gene expression by glucose has not been well studied. In the

present paper, we present two lines of evidence indicating that the human PDC E1 α gene is regulated by glucose, and that the increase in the E1 α mRNA level induced by glucose is due to increased transcription, providing the first evidence for PDC gene regulation at the transcriptional level.

First, in human HepG2 cells, nuclear run-on assays directly demonstrated an approx. 2-fold increase in E1 α gene transcription caused by glucose treatment. This increased transcription rate correlated well with the increased mRNA level induced by glucose, suggesting that the latter is due primarily to an increase in human E1 α (*PDHAI*) gene transcription. Secondly, the glucose-responsiveness of the human E1 α promoter demonstrated that the glucose effect is exerted through the transcription machinery. Two regions of the human E1 α promoter were found to be responsible for the glucose regulation; these represent variant forms of the ChoRE consensus sequence in the rat L-pyruvate kinase promoter, suggesting that glucose-mediated regulation of these genes may involve common specific transcription factor(s). The expression of the PDC E1 α gene in HepG2 cells is glucose-specific (Figure 5), whereas glucose had no stimulatory effect on the expression of the branched-chain oxo acid dehydrogenase α subunit mRNA in cultured human islet cells [38], indicating that high glucose concentrations may only affect the expression of enzymes involved in glucose/fatty acid metabolism.

The glucose stimulation of L-pyruvate kinase in primary liver cells is insulin-dependent and requires glucose concentrations greater than 10 mM [7,8]. In contrast, stimulation of PDC E1 α and E1 β mRNA levels in HepG2 cells by glucose occurs at 5.5 mM glucose, and is insulin independent (Figures 3 and 4). This difference may be attributed to the nature of the cell lines investigated, one being a primary cell line and the other a tumour cell line. A similar difference has been reported for the insulin-independent glucose (5 mM) stimulation of L-pyruvate kinase gene expression in mouse hepatoma mhAT3F cells [38a]. The difference in stimulatory glucose concentrations between primary and tumour cells has been attributed to the different expression of glucose transporter genes and hexokinase genes [38a,39,40]. Compared with normal hepatocytes, expression of the liver-specific GLUT2 and glucokinase genes is decreased in HepG2 cells, whereas expression of the GLUT1 and hexokinase I genes is elevated in HepG2 cells [41]. GLUT1 ($K_m \approx 2$ –5 mM) and hexokinase I ($K_m \approx 50 \mu\text{M}$) have a much higher affinity for glucose than GLUT2 ($K_m \approx 15$ mM) and glucokinase ($K_m \approx 15$ –20 mM), and their expression is not regulated by insulin. Therefore overexpression of GLUT1 and hexokinase I would cause the cell to become unresponsive to insulin and more sensitive to low glucose concentrations (a leftward shift) [39,40]. On the other hand, it has been shown that insulin alone cannot stimulate the expression of glucose-regulated metabolic enzyme genes even in primary cells [38a,41]. Insulin probably plays a permissive role in primary liver cells by stimulating the expression of GLUT2 and glucokinase [9,42]. Despite the loss of insulin dependence in HepG2 cells, the intracellular signalling pathway for glucose regulation is still intact, and hence the glucose effect on gene expression is still significant. The effect of glucose on E1 α expression is relatively small (2–2.5-fold increase in mRNA level) in HepG2 cells compared with that observed for glucokinase, but this may be attributed to the physiological regulation of PDC. Because PDC activity is also controlled acutely by covalent modification, an approx. 2-fold increase in total PDC activity can lead to a 2-fold increase in flux via PDC.

It has been observed that glucose must be metabolized in order to induce gene expression [4]. The fact that 2-deoxyglucose, but not 6-deoxyglucose, was able to partly stimulate acetyl-CoA

carboxylase gene expression suggests that at least the phosphorylation of glucose, the first step of glycolysis, is required for the glucose effect [44,45]. Glucose 6-phosphate has recently been suggested as a signal molecule for the effect of glucose on gene expression [4,46]. In the present study, fructose, galactose and pyruvate did not stimulate expression of the E1 α gene, because they all bypass the first step of glucose metabolism, namely the generation of glucose 6-phosphate. Fructokinase I activity levels in human and rat livers are approx. five and ten times higher respectively than the combined activities of glucokinase and hexokinase I in these tissues [47]. Fructokinase converts fructose into fructose 1-phosphate, which is then quickly converted into two three-carbon compounds, namely glyceraldehyde and dihydroxyacetone phosphate. Similarly, galactose is first converted into galactose 1-phosphate, which is subsequently converted into glucose 1-phosphate. Indeed, 6-deoxyglucose did not stimulate E1 α promoter activity (Figure 8). Despite the fact that pyruvate and fructose can acutely stimulate PDC enzyme activity [48], the regulation of PDC gene expression by glucose obviously is mediated by a signal generated by glucose. Besides glucose 6-phosphate, xylulose 5-phosphate derived from xylitol has also been claimed to be the signal molecule for the glucose stimulation of the L-pyruvate kinase promoter [37]. However, xylitol did not stimulate human E1 α promoter activity in HepG2 cells. Possibilities are that HepG2 cells have lost their normal metabolizing capacity for xylitol, or that a metabolite of xylitol is not a direct signal molecule for glucose regulation. In fact, a recent study indicated that only the production of glucose 6-phosphate correlates closely with the gene expression of S14 and fatty acid synthase [46].

The effect of glucose on E1 α gene transcription was abolished by okadaic acid at 100 nM (a concentration which inhibits both PP-1 and PP-2A), but not at 5 nM (a concentration which only specifically inhibits PP-2A), suggesting that the effect of glucose on human E1 α gene expression is related to a phosphorylation/dephosphorylation mechanism, possibly involving PP-1. It has been shown that dephosphorylation of transcription factor Sp1 by PP-1 is involved in the glucose-mediated stimulation of acetyl-CoA carboxylase promoter activity in adipocytes [32]. Also, inhibition of PP-1/PP-2A markedly inhibited glucose-stimulated insulin secretion in rat islets [49], indicating that the PP-1/PP-2A system may be involved generally in glucose-mediated processes.

The -100 bp promoter of the human E1 α gene is fully responsive to glucose (Figure 6), and mutations in the -83 to -61 bp region and in the -10 to -7 bp (CCGC) region resulted in reduced glucose-mediated stimulation (Figure 7). Sequence comparisons between the -83 to -61 bp region of the PDC E1 α gene and the ChoRE region of the L-pyruvate kinase and S14 genes reveals a consensus sequence, with identity of 11 out of 13 nucleotides with the L-pyruvate kinase promoter and of 8 out of 10 nucleotides with the S14 consensus sequence (Figure 7B). Although the ChoRE consensus sequence CACGTG is also the c-Myc/USF family transcription factor binding site, a single copy of this element does not confer glucose-responsiveness [50]. Either two copies of the perfect CACGTG sequence with a proper five-nucleotide space or multiple copies of a non-perfect CACGTG-related motif are required for maximum glucose-responsiveness [51]. Alternatively, an accessory factor binding site is required. As in the case of the L-pyruvate kinase promoter, the hepatic nuclear factor-4 binding site downstream of the ChoRE site is required for glucose-responsiveness [52]. These glucose-responsive elements usually confer 10–15-fold stimulation by glucose. Mutation of CACGTG to CCCGTG in the S14 promoter reduced glucose-responsiveness by about 50% [50], and mutation of the context sequence GTGGC to ACCGC reduced

glucose-responsiveness by about 40% [7]. The -83 to -61 bp region in the PDC E1 α promoter does not contain a perfect CACGTG sequence. The putative ChoRE site CCCCTG (-78 to -73 bp) sequence and its context sequence GACGC (-72 to -68 bp) show more resemblance to the mutated sequences mentioned above. Therefore it may not be surprising that the magnitude of glucose-responsiveness of the PDC E1 α promoter is much less (3–5-fold) than that of the L-pyruvate kinase and the S14 genes.

The identity of the ChoRE binding transcription factor(s) is still unknown. Although USF protein has been shown to bind this element *in vitro*, the involvement of USF has recently been ruled out by the overexpression of a dominant-negative form of USF in the study of the glucose regulation of the L-pyruvate kinase promoter [53]. However, c-Myc itself may be a candidate, as it can heterodimerize with many other transcription factors, and multiple c-Myc binding sites in a promoter may therefore behave differently from a single binding site. A recent report indicated that overexpression of c-Myc elevated glucose metabolism, including pyruvate kinase activity, and corrected the blood glucose concentration in streptozotocin-diabetic rats [54].

In summary, our results show that PDC E1 α mRNA levels are elevated by glucose in HepG2 cells, and that this is due to an increase in E1 α gene transcription. This conclusion is supported by studies on the promoter activity of the human E1 α (*PDH1*) gene. A ChoRE-like sequence in the human E1 α -100 bp promoter was found to be responsible for the effect of glucose on promoter activity. Treatment with 100 nM okadaic acid completely abolished the glucose effect on E1 α promoter activity, indicating that a dephosphorylation mechanism may be involved in the glucose-mediated stimulation of E1 α gene transcription. PDC plays a pivotal role in glucose oxidation, and the findings reported here show that glucose also exerts an effect on PDC gene transcription, especially as demonstrated for the E1 α gene. These findings represent an example of a mitochondrial enzyme being regulated at the transcriptional level by glucose.

We thank Dr. Murray Ettinger and Dr. Te-Chung Lee of this department and Dr. Ifeanyi Arinze (Meharry Medical College, Nashville, TN, U.S.A.) for their useful suggestions during the course of this study and for critical reading of the manuscript. This work was supported by National Institutes of Health grant DK20478.

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