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Insulin regulation of glucokinase gene expression: Evidence against a role for sterol regulatory element binding protein 1 in primary hepatocytes

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Abstract Liver key genes for carbohydrate and lipid homeostasis are regulated by insulin and glucose. The sterol regulatory-element binding protein-1c (SREBP-1c) has emerged as a mediator of insulin effects on gene transcription, particularly on glucokinase (GK). In this paper, we show that despite stimulation of GK promoter transcription by overexpression of mature SREBP-1c, insulin induced GK transcription at least 4 h ahead of accumulation of mature SREBP-1c in the nucleus. Importantly, the knockdown of *SREBP-1* mRNA using a RNA-interference technique reduced the level of nuclear SREBP-1 protein, diminished fatty acid synthase mRNA level, but did not affect GK and L-pyruvate kinase mRNA levels. We concluded that SREBP-1 is unlikely to be the mediator of the early insulin effect on GK gene transcription.

Keywords: Glucokinase; Small interfering RNA; Gene expression; Sterol regulatory element binding protein; Insulin

1. Introduction

Transcriptional activation of liver glycolytic and lipogenic genes, a relatively delayed phenomenon, requires the presence of both glucose and insulin, neither of which being active alone [1,2]. In contrast, stimulation of glucokinase (GK) gene, coding for the first enzyme involved in glucose metabolism, is a rapid strictly insulin-dependent and glucose-independent process [3,4]. When GK is overexpressed either in hepatoma cells [5] or in liver of diabetic mice [6], the transcriptional activation of glycolytic and lipogenic genes becomes insulin-independent and glucose-dependent. Similarly, in absence of GK in hepatic GK-KO mice, glycolytic and lipogenic genes are not induced after refeeding a high carbohydrate diet [7]. Therefore, insulin induction of GK gene expression is the key step to subsequent activation of glycolytic and lipogenic gene expression by glucose in liver. However, the molecular mechanisms by which insulin regulates GK gene expression are still controversial and the *cis*-acting elements mediating insulin effects remain unknown. The transcription factor sterol regulatory-element binding protein-1c (SREBP-1c), whose transcription is also insulin-dependent, was pointed out as the main mediator of insulin effect on GK gene expression [8,9], but other studies failed to confirm this finding [10,11]. Therefore, whether or not SREBP1-c mediates the action of insulin on GK gene expression is still a controversial issue.

Owing to the central role of insulin in the control of liver metabolism, and of GK in glucose metabolism, the goal of this study was to reinvestigate the role of SREBP-1c in mediating insulin effect upon GK gene expression. For this purpose, we studied in primary cultured hepatocytes the kinetics of insulin upregulation of GK gene transcription as well as the level of transcriptionally active SREBP-1 protein in the nucleus. Further, SREBP-1 knockdown by small interfering RNAs (siRNAs) resulted in an impaired induction of fatty acid synthase (FAS) gene in response to glucose and insulin, but did not prevent the induction of GK and L-pyruvate kinase (L-PK) genes. We clearly demonstrated that early insulin stimulation of GK gene was SREBP-1-independent.

2. Materials and methods

2.1. Primary hepatocyte culture

Hepatocytes were isolated from liver of 8-week-old male Wistar rats after perfusion with collagenase. Hepatocytes were seeded at a density of 3.5×10^6 cells per dish (20 cm^2) in M199 medium with Earl's salts (Invitrogen) supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 0.1% (w/v) BSA and 2% (v/v) Ultroser G (BioSepra). After a 4 h-incubation at 37 °C under 5% (v/v) CO_2, the cells were washed with PBS and switched for 16 h to a BSA–Ultroser-free medium. The medium was then supplemented with 1 μM dexamethasone, in the absence or presence of insulin and/or glucose as indicated in the figure legends.

2.2. Plasmid construction and transient transfection

GK promoter construct: The mouse GK–chloramphenicol acetyltransferase (CAT) plasmid is the PeCAT vector [12] in which the fragment (−960 to +19 bp) of the mouse liver GK promoter was subcloned, along with two fragments (+145 to +272 and +4035 to +4120) corresponding to the splice sequence in 5′ and 3′ of the first intron, respectively. These splice fragments were obtained by PCR using added restriction sites (underlined): For the 3′ splice: 5′-TATGGTACCCTTGGTGTGTGGTGGCTT-3′ and 5′-TTAGAGCTCTGCCAGGATCTGCTCTACC-3′. For the 5′ splice: 5′-TTACCTTGGAGCCCAGTCGTTGACTCT-3′ and 5′-TTCGGATCCTACAGGATCGCACTCA-3′. The PCR product for the 3′ splice fragment was

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Abbreviations: GK, glucokinase; L-PK, L-pyruvate kinase; FAS, fatty acid synthase; PEPCK, phosphoenolpyruvate kinase; SREBP, sterol regulatory element binding protein; CAT, chloramphenicol acetyltransferase; siRNA, small interfering RNA; SRE, sterol regulatory element; ChoRE, carbohydrate responsive element; ChREBP, carbohydrate responsive element binding protein

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digested by *Kpn1* and *Sst1*, and subcloned into PeCAT digested with the same enzymes. The PCR product for the 5′ splice and the promoter fragments were first digested by *Sty1* and then ligated together. The resulting fragment was digested by *Hind*III and *Bam*H1 and subcloned in the above-described plasmid digested with the same enzymes. All fragments generated by PCR were sequenced.

SREBP-1c expression plasmid: SREBP-1c plasmid was the pSV-Sport1-ADD1 (403) coding for the nuclear transcription factor [13] (gift of Dr. B.M. Spiegelman, Harvard Medical School, Boston, MA).

Transient transfections: Transfections were carried out as previously described [14]. In each experiment, 7.5 μg of the CAT plasmid and 2.5 μg of the luciferase plasmid were cotransfected. The luciferase standardization pRSV plasmid was used to monitor variations in transfection efficiency. The CAT assay and luciferase assay were performed as previously described [12].

2.3. RNA isolation and Northern-blot analysis

Total RNA extraction from cultured hepatocytes was performed using the RNAzol B reagent (Q Biogene) according to the manufacturer's instructions. 15 μg aliquots of total RNA were electrophoretically separated in denaturating formaldehyde agarose gels and transferred to Hybond N^+ membranes (Amersham). The cDNA probes were labeled with (α-^{32}P) dATP using random prime labeling (Amersham-Pharmacia Biotech). The membranes were hybridized with the indicated ^{32}P-labeled probe (10^6 cpm/ml) at 42 °C overnight. The GK, L-PK, phosphoenolpyruvate kinase (PEPCK), FAS and SREBP-1 probes were obtained as previously described [15]. Northern blots were hybridized with a ^{32}P-labeled ribosomal 18S probe to verify that equivalent amounts of total RNA were loaded in each lane.

2.4. Immunoblot analysis

Nuclear extracts and membrane fractions were prepared from cultured hepatocytes using the NE-PER™ Nuclear and Cytoplasmic Extraction Reagents Kit (Pierce) according to the instructions provided by the manufacturer. Protein concentration was determined using the Bradford method (Bio-Rad).

Proteins (35 μg) were separated by SDS–PAGE (7% polyacrylamide gel) and transferred to a nitrocellulose membrane (Bio-Rad). Immunoblot analysis was performed using a monoclonal anti-SREBP-1 antibody (clone 2A4 from NeoMarkers), a Lamin A/C antibody from Cell Signalling and the Enhanced Chemiluminescence System (Supersignal, Pierce).

2.5. Design, synthesis and transfection of siRNA

A target sequence in the rat SREBP-1 mRNA (GenBank Accession No. L16995) [13] was identified following the principles described by Elbashir et al. [16] as previously described [17]. Primary rat hepatocytes were cultured in antibiotic-free medium the day before transfection. Cells transfection was conducted using Lipofectamine 2000™ according to the supplier's instruction (Invitrogen). On day 1, hepatocytes were transfected with 200 pmol siRNA per 20-cm^2 dish. On day 2, the medium was supplemented with 100 nM insulin and 25 mM glucose. Twenty-four hours later, cells were harvested and either RNA or proteins were prepared.

3. Results

3.1. Regulation of GK gene transcription by SREBP-1c and insulin in cultured rat hepatocytes

Two potential binding sites for the transcription factor SREBP were identified, by computer analysis, in the mouse GK promoter (−113 to −106 and −65 to −55). To test SREBP-1c effect upon GK fusion gene expression we used primary cultured rat hepatocytes. We transiently transfected cells with a GK–CAT fusion gene, containing the promoter sequence located between −960 and +19, and either a control expression vector or the same vector encoding nSREBP-1c [13]. The cells were cultured in the absence or presence of insulin. When SREBP-1c was overexpressed, GK–CAT fusion gene expression was induced in a concentration-dependent manner (Fig. 1). At the maximum amount tested (1.25 μg), the control expression vector had no effect (data not shown) whereas the constitutively active SREBP1-c stimulated 2.5-fold the GK promoter activity. Strikingly, insulin treatment failed to induce basal GK–CAT fusion gene expression (Fig. 1A point 0 μg). These data indicated that the SREBP binding sites may well be functional but requestioned the role of SREBP-1c as a major mediator of insulin action on GK gene expression.

To ascertain these results, the well-established effects of insulin on endogenous GK mRNA level [3,18] were measured, in the same pool of cultured hepatocytes but untransfected. We observed the expected rapid (2 h) time-dependent accumulation of GK mRNA in the presence of insulin and low glucose concentration (5 mM) (Fig. 1B), indicating that insulin signaling

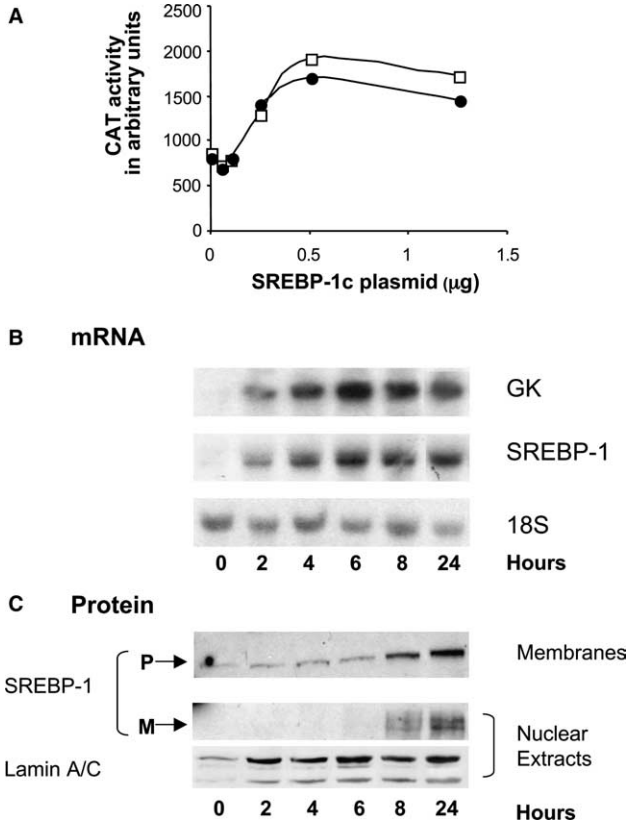

Fig. 1. Regulation of GK gene transcription by SREBP-1c and insulin in cultured rat hepatocytes. (A) Primary hepatocytes cultured for 16 h in the presence of 5 mM glucose without insulin were then transfected with 7.5 μg of CAT–GK fusion gene, 2.5 μg of the reference plasmid RSV luciferase and the indicated amount of expression vector for mature SREBP-1c. Cells were cultured in medium containing 5 mM glucose, in the absence (□) or presence (●) of 100 nM insulin. CAT activity was normalized according to transfection efficiency by measuring the luciferase activity. Values are means of four separate experiments performed in duplicate. (B) Time course of GK and SREBP-1 mRNA accumulation in hepatocytes cultured in the presence of 5 mM glucose and 100 nM insulin. Total mRNA (15 μg) were subjected to electrophoresis and blot hybridization with the indicated ^{32}P-labeled cDNA probe. (C) Time-course appearance of SREBP-1 precursor (P) and mature (M) proteins. Membrane pellet and nuclear extract (35 μg protein) were subjected to SDS/PAGE and analyzed by immunoblotting using an antibody against the precursor and mature forms of SREBP-1. Lamin A/C was used as control for the nuclear extract. The blots are representative of three independent experiments.