Molecular mechanism of hypoxia-mediated hepatic gluconeogenesis by transcriptional regulation

Jeong Hae Choi[a], Min Jung Park[a], Kook Whan Kim[a], Yoon Ha Choi[a], Sun Hee Park[a], Won Gun An[b], Ung Suk Yang[c], JaeHun Cheong[a,c,*]

[a] *Department of Molecular Biology, Pusan National University, Busan 609-735, Republic of Korea*
[b] *Department of Dentisty, Pusan National University, Busan, Republic of Korea*
[c] *Bongseng Memorial Hospital, Dong Gu, Busan 601-723, Republic of Korea*

Received 11 March 2005; accepted 27 March 2005

Available online 21 April 2005

Edited by Robert Barouki

Abstract **Until now, it is known that hypoxia increases the glycolytic enzyme expression at the transcriptional level. Here, we show evidence that hypoxia increases hepatic glucose output and HIF-1 and ATF-2-mediated transactivation of phosphoenolpyruvate carboxykinase (PEPCK), which plays a critical role as a rate-limiting enzyme in gluconeogenesis, gene in liver. HIF-1 directly bound to the specific PEPCK promoter region through its cognate binding element and found as an active complex with coactivator CBP. Additionally, ATF-2 was also involved to regulate hypoxia-dependent PEPCK transcription in the transcriptional complex with HIF-1 and CBP. Interestingly, retinoic acid (RA) signaling induced the recruitment of HIF-1 on the PEPCK promoter, resulting from the functional interaction of HIF-1 and ATF-2 with coactivator CBP. Taken together, these results suggest that hypoxia signaling leads the hepatic glucose production and release via the increased gene expression of gluconeogenic enzymes, possibly playing a role in providing glucose to other tissues, such as endothelial, brain and muscle cells.**

Keywords: HIF-1; ATF-2; Phosphoenolpyruvate carboxykinase; Gluconeogenesis; Hypoxia

1. Introduction

Liver is a key organ in the maintenance of systemic glucose homeostasis in mammals. The liver keeps blood glucose levels nearly constant under various nutritional conditions and provides a crucial source of fuel for the function of many organs and tissues under conditions of food deprivation. Deficient hepatic glucose output may lead to hypoglycemia and cause malfunction of key tissues and organs, such as the central nervous system, resulting in coma or death. On the other hand, elevated hepatic glucose secretion contributes very significantly to hyperglycemia in both type 1 and type 2 diabetes [1]. Glycogenolysis and gluconeogenesis are both key components of hepatic glucose output; suppression of hepatic gluconeogenesis has been shown to improve overall glycemic control in both human patients and type 2 diabetes animal models [2,3].

Changes in oxygen concentration in organisms represent a fundamental physiologic stimulus. In animals, this stimulus elicits both acute and chronic responses. Intracellular oxygen concentrations are maintained within a narrow range due to the risk of oxidative damage from excess oxygen (hyperoxia), and of metabolic demise from insufficient oxygen (hypoxia). Whereas acute responses often entail changes in the activity of preexisting proteins, chronic responses invariably involve changes in gene expression.

The phosphoenolpyruvate carboxykinase (PEPCK) promoter is a well-defined model for metabolic regulation of gene expression [4]. PEPCK, which catalyzes a regulatory step in gluconeogenesis, is expressed primarily in liver, kidney, small intestine, and adipose tissue, where its synthesis is regulated at the level of transcriptional initiation. In liver, dysfunctional regulation of the PEPCK promoter is associated with the pathophysiology of type 2 diabetes [5,6]. The PEPCK promoter integrates cues arising from diverse signaling pathways. PEPCK mRNA is induced by glucocorticoids, thyroid hormone, or glucagon [7], whereas insulin results in a repression of the promoter activities in a dominant manner [8]. The PEPCK promoter fragment encompassing −460 to +73 was demonstrated to be sufficient for hormonal regulation in liver, and many of the transcription factors that bind elements in this region have been identified [9,10]. Proteins demonstrated to bind and impact regulation of the PEPCK promoter include CREB, C/EBPα, C/EBPβ, ATF-2, NF1, HNF3, glucocorticoid receptor (GR), thyroid hormone receptor (TR), retinoic acid receptor (RAR), and retinoid X receptor (RXR). The energy balance state can affect signals for the PEPCK gene regulation through activating CREB, C/EBPα, and C/EBPβ, whereas ATF-2 mediates the stress response signals [10].

Most of the previous works on hypoxia-related glucose metabolism has been focused on glycolysis regulation, while our results showed that the hypoxia-mediated HIF-1 and ATF-2 transactivation play a critical role in glucose homeostasis through the increased gluconeogenic enzyme expression in hepatocytes. Here, we defined major *cis*-acting regulatory elements involved in the hypoxia-mediated expression of the PEPCK gene. Additionally, hypoxia and RA stimuli form an active transcriptional complex of HIF-1 and ATF-2, and CBP on the PEPCK promoter. These results suggest that hypoxia induces hepatic glucose output via a transcriptional regulation.

*Corresponding author. Fax: +82 51 513 9258.
E-mail address: molecule85@pusan.ac.kr (J. Cheong).

0014-5793/$30.00
doi:10.1016/j.febslet.2005.03.097

2. Materials and methods

2.1. Hepatocyte preparation

Livers of 15-day-old rats were perfused through the vena cava with a buffer consisting of: 140 mM NaCl, 2.6 mM KCl, 0.28 mM Na_2HPO_4/$2H_2O$, 5 mM glucose, and 10 mM HEPES (pH 7.4). The perfusion was first for 5 min with the buffer supplemented with 0.1 mM EGTA and then for 15 min with the buffer containing 5 mM $CaCl_2$ and 0.2 mg/ml collagenase type 2 (Worthington). The isolated hepatocytes were then washed and suspended in a small volume of DMEM (Gibco) without glucose and pyruvate and counted.

2.2. Release of neosynthesized glucose

Hepatocytes were incubated for 2 h at 37 °C with shaking in DMEM without glucose, but in the presence of 1 mM pyruvate, 10 mM lactate and 250 μM 3-isobutyl-1-methylxanthine. They were then pelleted, lysed in 0.1% SDS in PBS, and the protein content was determined. The glucose content of the cell lysate and the supernatant were measured by the glucose oxidase method.

2.3. Plasmids

HIF-1 cDNA was subcloned into pCMX1. pCMX1 was a gift from Catherine Thompson (Carnegie Institution of Washington) and was used in coupled transcription–translation reactions. In vitro translation products were verified by [^{35}S]Met incorporation and SDS–PAGE analysis. The reporter plasmids PEPCK-275 and PEPCK-543 were constructed by PCR amplification of rat genomic DNA encompassing positions −275 or −543 through +73 of the PEPCK promoter. The CRE1-mutant PEPCK-275 reporter, was prepared by standard mutagenesis, changing (−99) CCGGCCCCTT**ACG**TCAGAGGCG (−76) to CCGGCCCCTT**TTT**TCAGAGGCG.

2.4. Gel mobility shift analysis

Nuclear extracts were prepared from rat hepatocytes following with hypoxia treatment as indicated in the figure legends. Approximately 10 μg of nuclear extract was incubated with a probe. A double-stranded oligonucleotide encoding the PEPCK promoter sequence (promoter positions −149 to −128 for HIF-1 binding and −99 to −76 for ATF-2 binding) was used for gel shift analysis: 5′-GTTCCAAACCGTGCTGACCATG-3′ and 5′-CCGGCCCCTTA-CGTCAGAGGCG-3′, respectively. Binding reactions were assembled without probe and held 5 min on ice followed by 5 min at room temperature. Probe was added with further room temperature incubation for 30 min. Samples were separated in 4% acrylamide, 0.5× TBE [0.045 M Tris, 0.045 M boric acid, and 1.0 mM EDTA (pH 8.0)] gels run at 200 V constant voltage.

2.5. Transient transfection and luciferase assays

HepG2 cells were transfected by the standard calcium phosphate method. Cells were incubated with DNA precipitates for 16 h, washed, and maintained in complete medium 48 h prior to harvest. Relative luciferase and β-galactosidase activities were determined as described [10]. Basal promoter activity is reported as the activity observed after transfection of the reporter plus an appropriate amount of empty expression vector. In all cases, transfection data represent the means of three independent experiments.

2.6. Chromatin immunoprecipitation analysis

Cells were lysed for 5 min in L1 buffer (50 mM Tris, pH 8.0, 2 mM EDTA, 0.1% NP-40, and 10% glycerol) supplemented with protease inhibitors. Nuclei were pelleted at 3000 r.p.m. and resuspended in L2 buffer (50 mM Tris, pH 8.0, 0.1% SDS, and 5 mM EDTA). Chromatin was sheared by sonication, centrifuged and diluted 10 times in dilution buffer (50 mM Tris, pH 8.0, 0.5% NP-40, 0.2 M NaCl, and 0.5 mM EDTA). Extracts were pre-cleared for 3 h with 60 μl of a 50% suspension of salmon sperm-saturated protein A–agarose. Immunoprecipitations were carried out overnight at 4 °C. Immunocomplexes were collected with salmon sperm-saturated protein A for 30 min and washed three times (5 min each) with high-salt buffer (20 mM Tris, pH 8.0, 0.1% SDS, 1% NP-40, 2 mM EDTA, and 0.5 M NaCl) followed by three washes in no salt buffer (1× TE). Immunocomplexes were extracted in 1× TE containing 2% SDS, and protein–DNA cross-links were reverted by heating at 65 °C overnight. After proteinase K digestion, DNA was extracted with phenol–chloroform and precipitated in ethanol. About one-twentieth of the immunoprecipitated DNA was used in each PCR. Quantitative duplex PCR assay was performed to analyze the amount of DNA precipitated by specified antibodies in proportion to input DNA. Two pairs of primers were used: Forward (5′-AAGTTTAGTCAATCAAACGTT-3′) and Reverse (5′-TGCTTGGTAGCTAGCCCTCCT-3′) for the PEPCK promoter. The PCR conditions were as follows: 1.25 U of *Taq* DNA polymerase (Amersham Biosciences), 100 ng of each primer, 200 μM dNTP, 2.5 μl of 10× *Taq* buffer and double-distilled water to a final volume of 25 μl: 94 °C for 180 s; 34 cycles at 94 °C for 45 s, 60 °C for 60 s and 72 °C for 60 s; final elongation at 72 °C for 10 min.

3. Results

3.1. Hypoxia increases glucose output in hepatocytes and HepG2 cells

It is known that hypoxia increases the glucose utilization in many cell types for providing ATP. However, for the sufficient supply of the entire glucose amount to some cells including muscle, vascular endothelial and brain cells, it is required for maintaining whole glucose homeostasis through hepatic glucose production. To examine whether hypoxia regulates the hepatic glucose output, we measured the glucose release from rat hepatocytes and human hepatoma cell line HepG2. As shown in Fig. 1, hypoxia stimuli (2% oxygen concentration) induced the glucose release in both isolated rat hepatocytes and cultured HepG2 cells with 45% and 48% increase compared to normoxia condition (12% oxygen concentration), respectively. Together, these findings suggested that the hypoxia stimuli might have an important role in the biology of endogenous glucose production and release from hepatocytes.

3.2. Hypoxia increases transcriptional activation of PEPCK promoter by HIF-1 activation

To explore the role of hypoxia stimuli in the regulation of the hepatic glucose production pathway, we determined the effect of hypoxia in the gene expression of PEPCK, which is a

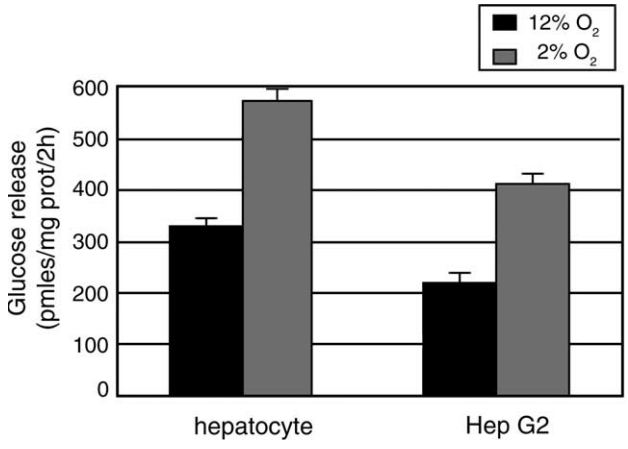

Fig. 1. Hypoxia increases glucose output in hepatocytes. Isolated rat hepatocytes and human hepatoma cell line HepG2 were incubated for 2 h at 37 °C in DMEM without glucose but in the presence of 1 mM pyruvate and 10 mM lactate. The data are the mean ± SEM of four experiments. Hepatocytes and HepG2 cells were incubated for 3 h in the normoxia (12% O_2) or in the hypoxia condition (2% O_2).