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Detailed molecular analysis of the induction of the L-PK gene by glucose

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Abstract

Glucose has powerful effects on gene expression and participates in the fasted to fed transition of the liver. However, the molecular mechanism of glucose-regulated gene expression has not been completely described. In the present study, we performed a detailed analysis of the molecular events of the insulin-independent glucose response of the liver-type pyruvate kinase (L-PK) gene. L-PK mRNA was increased by glucose at the transcriptional level as determined by real-time RT-PCR, mRNA stability measurements, and nuclear run-on assays. LY294002 and LY303511 inhibited the glucose response of the L-PK gene at the transcriptional level. Histones H3 and H4 associated with the L-PK gene promoter were hyperacetylated and HNF4α was constitutively bound in low and high glucose. Treatment with 20 mM glucose increased recruitment of ChREBP, additional HNF4α, and RNA polymerase II. Glucose stimulated the phosphorylation of the C terminal domain of RNA polymerase II, with increased Ser5 phosphorylation near the transcription start site and increased Ser2 phosphorylation near the termination signal. LY294002 and LY303511 blocked the recruitment of RNA polymerase II to the L-PK gene, reducing the rate of transcription. The results of these studies demonstrate fundamental details of the molecular mechanism of glucose activated gene expression.

Keywords

L-type pyruvate kinase; LY294002; LY303511; insulin-independent; glucose signaling; hepatocytes; chromatin immunoprecipitation; gene promoter

Introduction

During the fasted-to-fed transition, insulin and glucose work in concert to bring about a programmed change of gene expression in the liver. This phenotypic switch includes an increase in glycolytic and lipogenic gene expression and a decrease in gluconeogenic and ketogenic gene expression [1]. Insulin is required for glucokinase expression, which in turn is required for increased glucose uptake and signaling. By expressing glucokinase in the absence

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of insulin, such as with an adenovirus, one can separate the effects of insulin and glucose on gene expression [1,2]. One gene that is induced by increased glucose flux in the absence of insulin is L-type pyruvate kinase (L-PK) [2]. Glucose activates the L-PK gene by recruiting carbohydrate response element binding protein (ChREBP). In the fasted state, cAMP-dependent protein kinase phosphorylates ChREBP, rendering it unable to bind to DNA and sequestering it in the cytosol [3]. After a meal rich in carbohydrates, the increase in glucose flux leads to an increase in X-5-P levels, which in turn leads to increased PP2A activity. This PP2A dephosphorylates and activates ChREBP, leading to increased glycolytic and lipogenic gene expression [3], though the details of this model have been challenged [4;5;6].

In the present study we sought to more completely describe the molecular details of the glucose response. Using real-time RT-PCR, nuclear run-ons, and chromatin immunoprecipitation, we performed a detailed molecular analysis of the insulinin-dependent glucose response of the L-PK gene.

Materials and Methods

Cell Culture

HL1C rat hepatoma cells [7] were maintained as previously described [8]. Cells were transduced with an adenovirus expressing glucokinase (Ad-GK [9]) (a gift from Dr. Christopher Newgard) and incubated for 24 h. The amount of Ad-GK required to facilitate a glucose response in the HL1C hepatomas was established empirically by a functional titration, wherein the amount of Ad-GK that conferred a 2 to 3 fold increase in L-PK gene expression was determined. Primary hepatocytes were isolated by a perfusion method, as previously described [10].

Reverse transcription-polymerase chain reaction (RT-PCR) analysis

RNA was isolated from HL1C hepatoma cells and primary hepatocytes using Tri-Reagent (Molecular Research Center, Cincinnati, OH) according to the manufacturer's instructions. RT-PCR was carried out using a SYBR green master mix (Bio-Rad, Hercules, CA) in an Applied Biosystems Prism 7300 Real-Time PCR System as previously described [6]. Fold change in mRNA expression was determined using the ΔΔcT method, with all genes normalized to cyclophilin [11].

Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) was performed as previously described [10]. The ChREBP antibody was purchased from Novus (Littleton, CO, #NB400-135); the anti-rabbit IgG (#sc2027) and HNF4α (#sc8987) antibodies were purchased from Santa Cruz Biotechnologies (Santa Cruz, CA); the RNA polymerase II (#05–623), acetylated histone H3 (#06–599), and acetylated histone H4 (#06–866) antibodies and anti-mouse IgG (#12–371) were purchased from Millipore (Billerica, MA). The RNA polymerase II CTD phospho-Ser5 (ab5131) and phospho-Ser2 (ab5095) antibodies were purchased from Abcam (Cambridge, MA).

Nuclear Run-on

Nuclear run-ons were performed using a slightly modified version of the method described by Patrone et al. [12]. PCR was performed as described above. The primer sequences for the PCR reactions were as follows (upstream and downstream, respectively): β-actin, 5′-tagccctcttttgtgccttg-3′, 5′-tgccactcccaaagtaaagg-3′. L-PK, 5′-gaacacctctgccttctgga-3′, and 5′-ccctgcacaaatctcacaaa-3′.

Statistical analysis

All values are expressed as ± S.E.M. Data analyses were performed with SPSS 15.0 for Windows. A one-way ANOVA was performed to detect statistical differences ($P \leq 0.05$). Differences within the ANOVA were determined using a Tukey's post hoc test.

Results and Discussion

Glucose increases L-PK gene expression at the level of transcription

A 5 h or 16 h treatment with 20 mM glucose increased L-PK mRNA levels in glucokinase-expressing HL1C cells and primary hepatocytes, respectively, as expected (Figure 1A and B). This increase was found to be exclusively due to transcription, as mRNA turnover was not affected by glucose (whether the cells were pretreated with glucose or not), and a nuclear run-on assay demonstrated that glucose increased the rate of transcription of the L-PK, but not the β-actin gene (Figure 2A and B). A screen of possible inhibitors of the glucose response revealed that two highly related compounds, LY294002 and LY303511, both inhibited the glucose-stimulated increase of L-PK mRNA in HL1C cells and in primary hepatocytes (Figure 1A and B). This inhibition was at the level of transcription as the stability of L-PK mRNA was unaffected and the rate of transcription was reduced to basal levels by these compounds (Figure 2A and B).

Silencing PI3-K does not prevent the glucose-mediated induction of L-PK mRNA

Since LY294002 is known to inhibit PI3-K, we tested if the inhibition of PI3-K, by either the specific inhibitor wortmannin, or by the silencing of the p110β catalytic subunit of PI3-K (si-p110β), the insulin-sensitive isoform of p110 [13], using small interfering RNA (siRNA) affected the glucose-mediated induction of L-PK mRNA. Neither wortmannin (data not shown) nor si-p110β significantly altered glucose-stimulated L-PK mRNA levels compared to controls (Supplement Figure 1). Thus, the inhibition of the L-PK glucose response by LY294002 and LY303511 is most likely a PI3-K-independent effect.

Effect of inhibiting non-"traditional" target pathways of LY294002 and LY303511 on the glucose response of the L-PK gene

LY294002 and LY303511 inhibit a number of enzymes in a PI3-K-independent manner [14; 15], including the protein kinase mTOR, protein kinase CK2 [16;17], and voltage-gated K^+ channels (K_v channels) in pancreatic β-cells [18;19]. We found that rapamycin, an inhibitor of mTOR, and tetraethyl ammonium (TEA), an inhibitor of K_v channels [18], did not blunt the glucose-mediated induction of the L-PK gene (Supplementary Figure 2 and Supplementary Figure 3). We also found that apigenin and 4,5,6,7-tetrabromobenzotriazole (TBB), known inhibitors of CK2 did inhibit L-PK mRNA levels, but appeared to do so in a non-specific manner, and therefore inhibition of protein kinase CK2 did not mimic the inhibition of the L-PK gene by LY294002 and LY303511 (data not shown).

Glucose recruits the binding of ChREBP to the L-PK gene promoter

We next examined the effects of LY294002 and LY303511 on the molecular events associated with the glucose-mediated activation of the L-PK gene. The transcription factors ChREBP and HNF4α are required for the glucose-mediated induction of the L-PK gene in hepatocytes [20; 21]. ChREBP forms a heterodimer with its partner Mlx and binds to the carbohydrate response element (ChoRE) located between −171 to −142 relative to the transcription start site of the L-PK gene [22]. HNF4α binds to a DR-1 element in the L-PK gene promoter located from −144 to −126 bp relative to the transcription start site [23]. A chromatin immunoprecipitation (ChIP) assay was used to determine the effect of glucose on the L-PK promoter occupancy of ChREBP and HNF4α. When primers for the L-PK promoter region flanking the ChoRE were

used, no ChREBP was detected from cells incubated with 2 mM glucose, but ChREBP was recruited to the region in the presence of 20 mM glucose (Figure 3A). The addition of LY294002 or LY303511 had no significant effect on ChREBP binding to the L-PK gene promoter. As a control, no difference in signal was detected when primers for the coding region of the L-PK gene (6000 bp downstream from the transcription initiation site) were used.

HNF4α was bound to the L-PK promoter from cells treated with 2 mM glucose, which increased in cells exposed to 20 mM glucose, but the difference did not reach significance (Figure 3B, p=0.117). These results are consistent with a previous study [24]. LY294002 and LY303511 did not inhibit the binding of HNF4α to the L-PK gene promoter.

The L-PK gene promoter is hyperacetylated in the absence and presence of glucose

Histone acetylation often increases prior to or concomitant with gene activation [25;26]. To determine patterns of histone acetylation along the L-PK gene, we performed a ChIP analysis using antibodies against acetylated histones H3 and H4. In this experiment, primers were used for regions of the L-PK gene that flanked sequences centered at −500, −165, 0, +500, +1200, and +6000 bp relative to the transcription start site. The region between −500 and +500 of the L-PK gene was hyperacetylated when compared to downstream regions. No difference was seen in the levels of acetylated histone H3 (Figure 3C) or histone H4 (Figure 3D) bound to the L-PK gene between cells treated with either 2 or 20 mM glucose. LY294002 and LY303511 had no effect on the binding of these histones to the gene, with the exception of a small but statistically significant increase in histone H4 binding at the +6000 region (Figure 3D).

Glucose promotes and LY294002 and LY303511 prevents the recruitment and subsequent phosphorylation of RNA polymerase II on the L-PK gene

We next examined the effects of glucose on the recruitment of RNA polymerase II (Pol II) to the L-PK gene using the ChIP assay. At every region examined, the binding of Pol II was significantly increased in cells treated with 20 mM glucose when compared to control cells (Figure 4A). Further, at every region LY294002 and LY303511 reduced the glucose-stimulated binding of Pol II to the L-PK promoter.

The C-terminal domain (CTD) of RNA Pol II contains between 25 and 52 copies of a 7 amino acid repeat, $Y_1S_2P_3T_4S_5P_6S_7$ [27]. Phosphorylation of two of the serines, Ser2 and Ser5, is required for RNA processing, including the addition of the 5' CAP and 3' polyadenylation [28;29;30]. After Pol II is recruited to a promoter, it becomes phosphorylated at Ser5 by the TFIIH kinase CDK7 [31]. As Pol II proceeds during elongation, Ser2 becomes phosphorylated by CDK9 [32]. When Pol II approaches termination, Ser5 becomes dephosphorylated by an as-yet unidentified phosphatase. Thus, there is typically more Pol II phosphorylated at Ser5 bound to the 5' end of an activated gene and more Pol II phosphorylated at Ser2 bound to the 3' end [31].

A ChIP assay was performed with antibodies specific for either Pol II $pCTD^{Ser5}$ or Pol II $pCTD^{Ser2}$ to determine if glucose affected the phosphorylation pattern of the Pol II CTD. There was a significant increase between 2 and 20 mM glucose in the amount of Pol II $pCTD^{Ser5}$ bound at every region of the L-PK gene examined (Figure 4B), with more bound to the promoter region than the coding region. Twenty mM glucose also caused a significant increase in the amount of Pol II $pCTD^{Ser2}$ bound to the regions centered at −500 and +6000 from the initiation site (Figure 4C). The addition of LY294002 or LY303511 reduced the amount of both Pol II $pCTD^{Ser5}$ and Pol II $pCTD^{Ser2}$ bound to the L-PK gene, but the reduction in phosphorylation was apparently equivalent to the reduction in total Pol II, suggesting that these compounds had no specific effect on CTD phosphorylation.

In summary, LY294002 and LY303511 did not inhibit the recruitment of ChREBP or HNF4α to the L-PK promoter, nor did they alter histone acetylation, but they did reduce the glucose-mediated recruitment of RNA polymerase II to the L-PK gene.

Summary and Conclusions

Together these data provide a composite picture of the molecular events at the L-PK gene after exposure to glucose as follows: the gene is expressed constitutively at a low rate of transcription with HNF1 (data not shown) and HNF4α bound regardless of glucose concentration, and the gene is hyperacetylated from between −500 to +500 under all conditions. In the presence of glucose, ChREBP, Pol II, and to a lesser extent, HNF4α, are recruited to the gene. The Pol II CTD is phosphorylated on Ser5, processes through the gene as it transcribes nascent preRNA, and is phosphorylated at Ser2 as it approaches the end of the coding sequence.

This detailed description of the glucose response of the L-PK gene allowed us to discriminate between various possible ways in which LY294002 and LY303511 might inhibit the transcription of the L-PK gene. LY294002 and LY303511 did not inhibit the recruitment of ChREBP or HNF4α to the L-PK promoter, nor did they alter histone acetylation, but they did reduce the glucose-mediated recruitment of RNA polymerase II to the L-PK gene. We found that LY294002 and the closely related LY303511, which does not inhibit PI3-K, had identical (and PI3-K-independent) effects on the transcription of the L-PK gene. These effects did not appear to be related to known non-PI3-K inhibitory effects of these compounds. Thus, the present study provides yet another case to proceed with interpretative caution with regard to the pervasive perception in the literature that LY294002 is exclusively an inhibitor of PI3-K.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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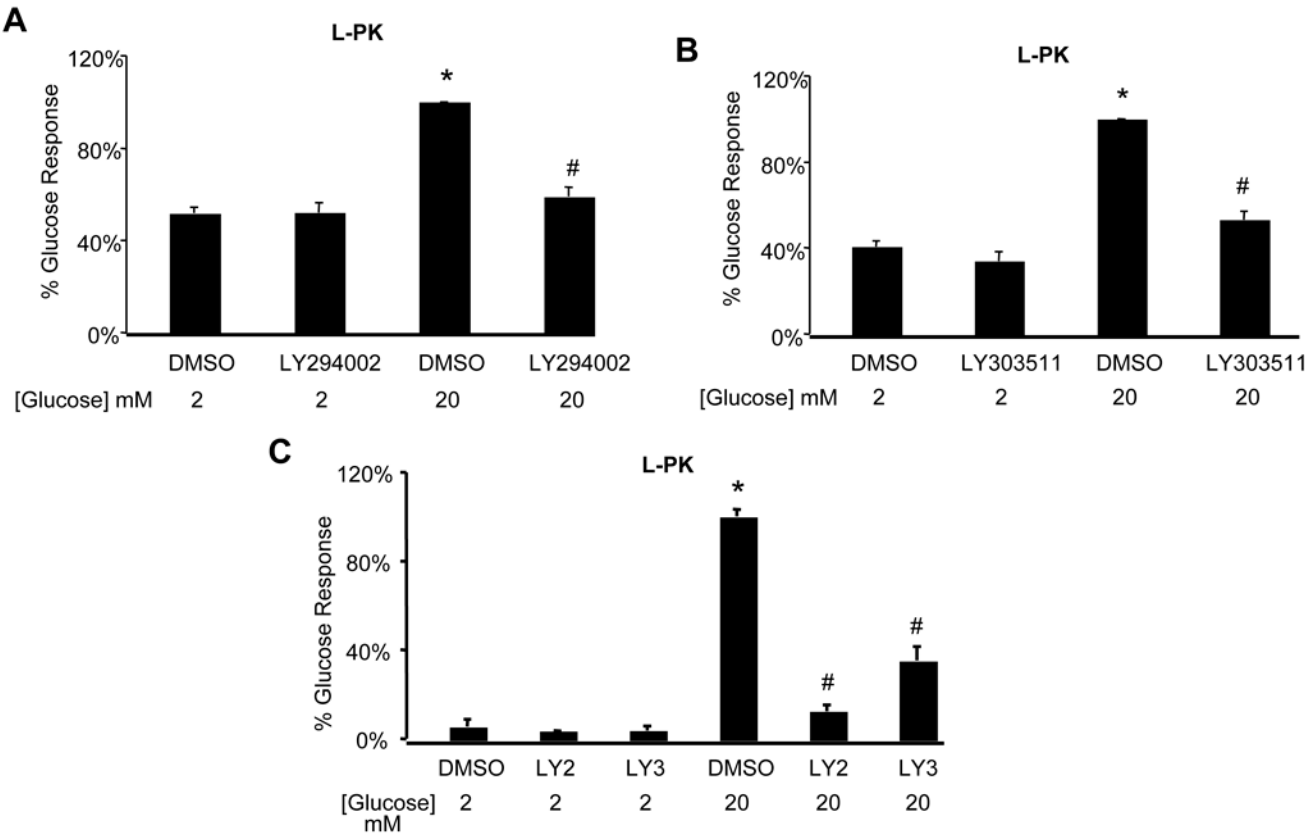

Figure 1. Glucose increases L-PK mRNA levels

HL1C cells expressing glucokinase were treated in 2 mM or 20 mM glucose with 50 μM LY294002 (**A**) or LY303511 (**B**). After a 5 h treatment, RNA was harvested from the cells and real-time RT-PCR was performed. The relative difference in L-PK mRNA levels between the control cells at 2 and 20 mM glucose was calculated using the ΔΔcT method [11] (normalized to cyclophilin) (n = 3; ± SEM; **p* < 0.05 vs. 2 mM glucose + DMSO control, #*p* < 0.01 vs. 20 mM glucose + DMSO control) (**C**) Primary rat hepatocytes were treated for 16 h in serum-free media with 2 mM or 20 mM glucose either DMSO, 50 μM LY294002, or 50 μM LY303511. RNA was harvested and real-time RT-PCR performed using primers specific for the coding region of the L-PK gene. The data are presented as the percent of the normal glucose response, which was calculated as the relative difference between the DMSO-treated 2 mM glucose sample and the vehicle-treated 20 mM glucose sample. (n = 4; ± SEM; **p* < 0.05 vs. 2 mM glucose + DMSO control, #*p* < 0.05 vs. 20 mM glucose + DMSO control)

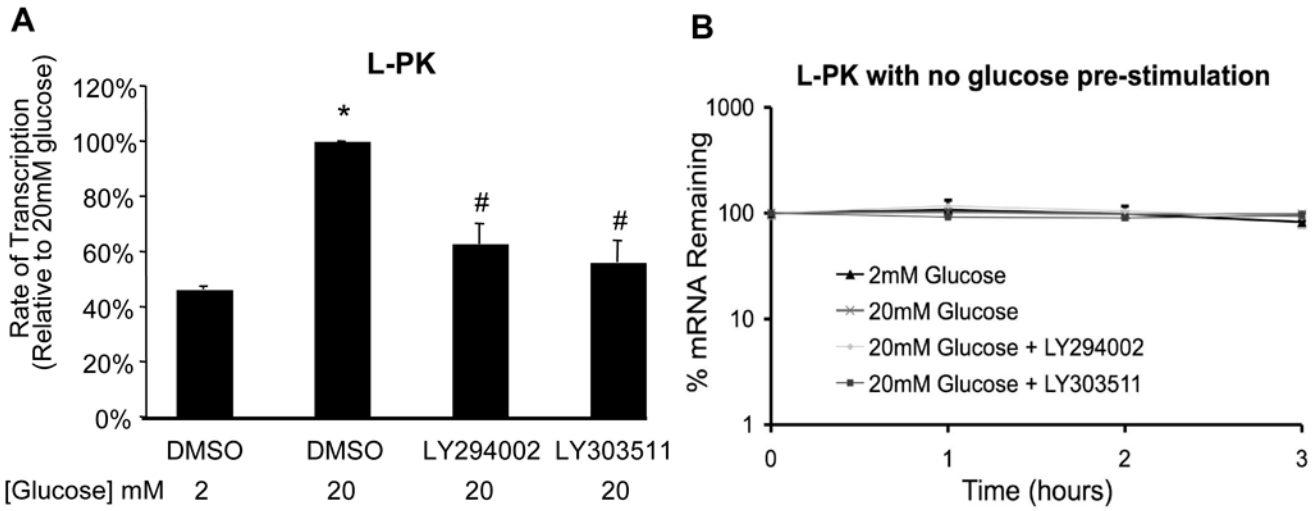

Figure 2. Glucose increases the transcriptional rate of the L-PK gene and does not affect L-PK mRNA stability

(**A**) Glucokinase-expressing HL1C cells were treated in 2 or 20 mM glucose with DMSO, 50 μM LY294002, or 50 μM LY303511 for 5 h. Nuclei were isolated and incubated in the presence of biotinylated UTP and transcription was allowed to proceed for 20 min. The biotinylated hnRNA was used as a template in real-time RT-PCR using primers specific for nascent L-PK mRNA. The relative difference in L-PK hnRNA levels between 2 and 20 mM glucose was set as 100%. (n = 3; ± SEM; *$p < 0.005$ vs. 2 mM glucose + DMSO control, #$p < 0.05$ vs. 20 mM glucose + DMSO) (**B**) HL1C cells transduced with Ad-GK were treated with 10 μg/mL of the transcription inhibitor actinomycin D and either 2 or 20 mM glucose with DMSO, 50 μM LY294002, or 50 μM LY303511. RNA samples were collected at 0, 1, 2, and 3 h. Real-time RT-PCR was performed, and the relative difference in L-PK mRNA levels at the various time points was calculated relative to the 0 h time point. (n = 3, ± SEM)

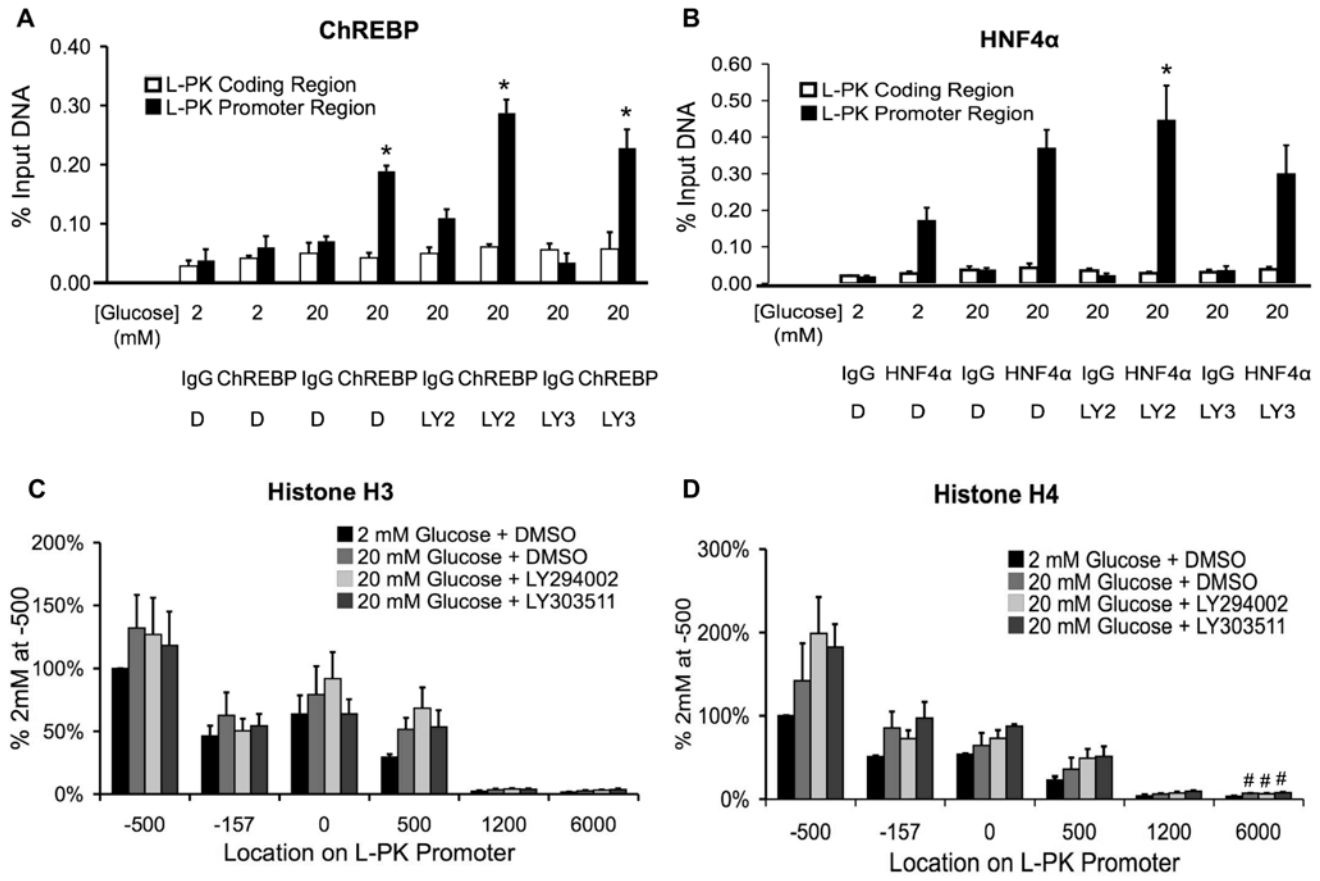

Figure 3. Glucose recruits ChREBP to the L-PK gene promoter

HL1C rat hepatoma cells were transduced with an adenovirus expressing glucokinase. Cells were treated with 2 or 20 mM glucose with DMSO, 50 μM LY294002 or 50 μM LY303511 for 4 h. Chromatin was isolated and fragmented, and ChIP was performed with control IgG or antibodies directed against ChREBP (**A**), HNF4α (**B**), or the acetylated forms of histone H3 (**C**) or H4 (**D**). Primers centered on each side of −500, −157 (which includes the ChoRE), 0, +500, +1200, and +6000 bp relative to the transcription start site of the L-PK gene were used for real-time PCR. The data are presented as relative to 100% of the signal from the sample treated in 2 mM glucose with DMSO from the L-PK coding region (ChREBP and HNF4α) or −500 bp amplicon (H3 and H4) after subtraction of the background IgG signal. [n = 3 (ChREBP), n = 4 (HNF4α), ± SEM, **p* < 0.05 vs. 2 mM glucose with DMSO and antibody (ChREBP or HNF4α)]

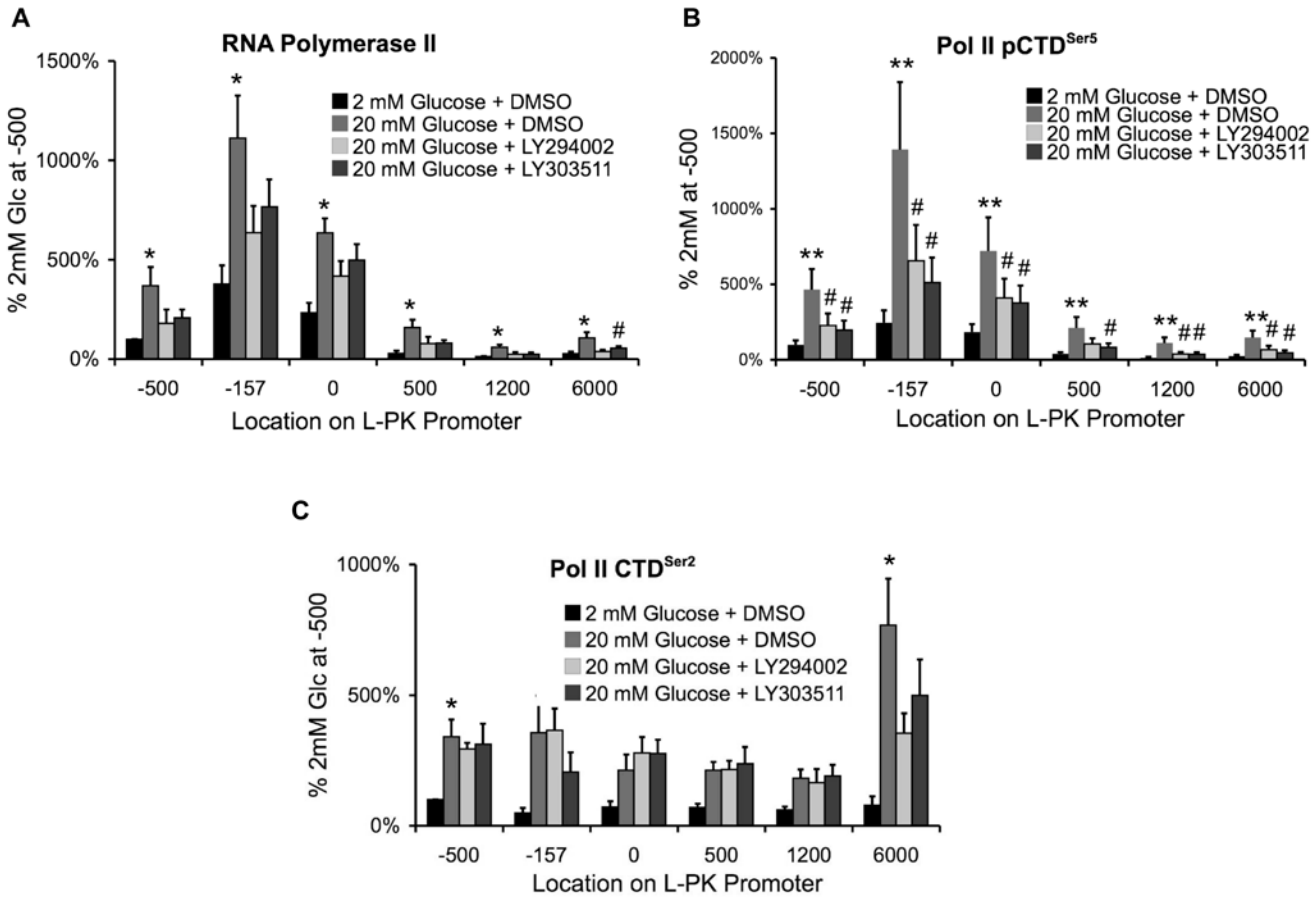

Figure 4. Glucose promotes while LY294002 and LY303511 inhibits the recruitment of RNA polymerase II to the L-PK gene

Glucokinase-expressing HL1Cs were treated in 2 or 20 mM glucose with DMSO, 50 μM LY294002 or 50 μM LY303511 for 4 h. A ChIP assay was performed using control IgG or antibodies directed against the CTD of RNA polymerase II (**A**), phosphorylated CTD^{Ser5} (**B**), or phosphorylated CTD^{Ser2} (C). Primers flanking L-PK gene regions −500, −157 (including the ChoRE), 0, +500, +1200, and +6000 bp relative to the transcription start site of the L-PK gene were used for real-time PCR. The data are presented as relative to 100% of the control signal (2 mM glucose with DMSO) generated at the −500 bp region after subtraction of the background IgG signal. (n = 3–5, ± SEM, *$p < 0.05$ vs. 2 mM glucose + DMSO, **$p < 0.01$ vs. 2 mM glucose + DMSO, #$p < 0.05$ vs. 20 mM glucose + DMSO)