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FEBS Letters 340 (1994) 221–225

FEBS 13734

FEBS LETTERS

Insulin inhibits glucocorticoid-induced stimulation of liver 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase gene transcription

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Received 14 January 1994

Abstract

6-Phosphofructo-2-kinase (PFK-2)/fructose-2,6-bisphosphatase (FBPase-2) catalyzes the synthesis and degradation of fructose 2,6-bisphosphate, a potent stimulator of glycolysis. Transcription of the mRNA encoding rat liver PFK-2/FBPase-2 is stimulated by insulin and by glucocorticoids in rat hepatoma cells. We show here that insulin can also prevent and reverse this glucocorticoid effect. The inhibitory effect of insulin is independent of extracellular glucose and does not require ongoing protein synthesis. We conclude that insulin exerts opposite effects on PFK-2/FBPase-2 gene transcription depending on the hormonal context.

Key words: Fructose-2,6-bisphosphatase; Glucocorticoid hormone; Insulin; 6-Phosphofructo-2-kinase; Transcription; Rat liver

1. Introduction

The pace of glycolysis and gluconeogenesis is regulated in liver by the availability of substrates, and by short-term and long-term control mechanisms [1]. Long-term control mainly involves the expression of genes encoding glycolytic and gluconeogenic enzymes. Hormones play an essential role in the coordinate expression of these genes [1], and a full understanding of their regulation requires knowledge of the molecular mechanisms of hormone action. The bifunctional enzyme 6-phosphofructo-2-kinase (PFK-2, EC 2.7.1.105)/fructose-2,6-bisphosphatase (FBPase-2, EC 3.1.3.46) catalyzes the synthesis and degradation of fructose 2,6-bisphosphate which, by stimulating 6-phosphofructo-1-kinase and inhibiting fructose-1,6-bisphosphatase, is a potent stimulator of glycolysis and inhibitor of gluconeogenesis. There are several PFK-2/FBPase-2 isozymes with differing expression depending on the tissue. The liver-, muscle- and fetal-type isozymes are encoded by the same gene which contains 17 exons [2,3]. The liver (L), muscle (M) and fetal (F) mRNA each originate from a distinct promoter and share exons 2–14, but contain in addition one or more specific exons at their 5′ end (Fig. 1). The exon specific for the L mRNA contains 346 base pairs and is called exon 1L.

The activity of the L promoter is controlled by ubiquitous and liver-specific transcription factors [4,5] and by a glucocorticoid responsive unit located in the intron downstream of exon 1L [6]. Transcription from the L promoter increases upon glucocorticoid treatment of intact rats [7] and of cultured rat hepatoma Fao cells [8]. It also increases in the liver of diabetic rats treated with insulin [9,10] and in Fao cells exposed to insulin [8], in keeping with the stimulation of glycolysis by insulin [1]. However, the effect of insulin on the enzymes involved in carbohydrate metabolism is usually opposite to that of glucocorticoids [1]. We have re-examined the control exerted by these hormones on L mRNA in rat hepatoma cells and show that, in fact, insulin opposes the stimulatory effect of the glucocorticoid dexamethasone.

2. Experimental

2.1. Cells

Rat hepatoma FTO-2B cells were grown as monolayers on 60-mm dishes in DMEM/Ham's F12 medium supplemented with 10% foetal calf serum and used in the logarithmic phase of growth. To test the effect of glucose, RPMI medium containing no glucose or 2 g of glucose per l was used instead of DMEM/Ham's F12. In some experiments, serum was replaced by a solution of 1% bovine serum albumin in phosphate buffered saline (final albumin concentration in the medium was 0.1%). Dexamethasone was dissolved in ethanol. Insulin was porcine (Actrapid MC, Novo).

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Abbreviations: FBPase-2, fructose-2,6-bisphosphatase; PFK-2, 6-phosphofructo-2-kinase; RT-PCR, reverse transcription-polymerase chain reaction.

SSDI 0014-5793(94)00119-G

2.2. Plasmids

pT7.3/388 was constructed by inserting in the *Xba*I and *Sma*I sites of pSP72 (Promega) a 385-bp *Xba*I–*Stu*I genomic fragment of the PFK-2/FBPase-2 gene A [2]. This genomic fragment (positions 3–388 with respect to the L mRNA cap site) encompasses the liver-specific exon 1L. pT7.3/388ΔR is identical to pT7.3/388 except for a 64-bp *Rsa*I *Rsa*I deletion in the insert.

2.3. Competitive reverse transcription-polymerase chain reaction (RT-PCR)

Total cytoplasmic RNA from 10^6 to 4×10^6 FTO-2B cells (test RNA) was isolated as follows. The cells were washed and pelleted in phosphate buffered saline, and lysed for 5 min on ice in 200 μl of a solution containing 10 mM Tris-Cl (pH 8.6), 140 mM NaCl, 1.5 mM $MgCl_2$, 1 mM dithiothreitol, 0.5% NP-40 and 1,000 U/ml human placental ribonuclease inhibitor. The lysate was centrifugated for 1 min at $10,000 \times g$ and the supernatant was added to 200 μl of a solution containing 200 mM Tris-Cl (pH 7.4), 25 mM EDTA, 300 mM NaCl, 2% SDS and 100 μg/ml proteinase K. After a 30-min incubation at 37°C, the solution was extracted with phenol/chloroform and the RNA was precipitated with isopropanol. The RNA pellet was resuspended and incubated for 1 h at 30°C in 50 μl of a solution containing 100 mM sodium acetate (pH 5.0), 5 mM $MgSO_4$, and 3 U RNase-free DNase I (Promega) to remove traces of cellular DNA. After phenol/chloroform extraction the RNA was precipitated with isopropanol, resuspended in double distilled water and quantified by spectrophotometry. The integrity of the RNA preparations was checked by visualizing the 28 S and 18 S rRNA on agarose gels. Internal standard RNA was synthesized in vitro by transcribing with T7 RNA polymerase the pT7.3/388DR plasmid linearized with *Pvu*II. This yielded a 370 base RNA that was treated with DNase I under the same conditions as the test RNA. This RNA encompasses nucleotides 3–388 of exon 1L but contains a 64-bp deletion between nucleotides 59 and 124. Reverse transcription with random hexamers and PCR reactions were performed with a RNA-AMP kit (Perkin Elmer-Cetus) under the conditions described by the manufacturer. Each reaction contained 500 ng of test RNA and 0.04 pg of internal standard RNA. After reverse transcription, the test and internal standard cDNAs were competitively co-amplified by PCR with primers that hybridize to nucleotides +32 to +49 and to nucleotides +284 to +305 (Fig. 1). This yielded products of 274 and 210 bp corresponding to amplified exon 1L and internal standard, respectively. The 5′–3′ sequence of the PCR primers was GGCTCTCAGCACCCACAT (5′ primer) and GGAATCCAGATCTTCTGTAACC (3′ primer). For PCR 30 cycles were performed at 94°C (1 min), 51°C (1 min) and 72°C (1 min). To check for RNA or DNA contaminations RT-PCR was performed either in the absence of RNA or in the absence of reverse transcriptase. The quantification of the RT-PCR reactions was performed by adding 2 μCi of [α-^{32}P]dCTP during the amplification. The PCR products were separated on 2% agarose gels from which the bands were cut out and counted by scintillation. All experiments were performed at least twice.

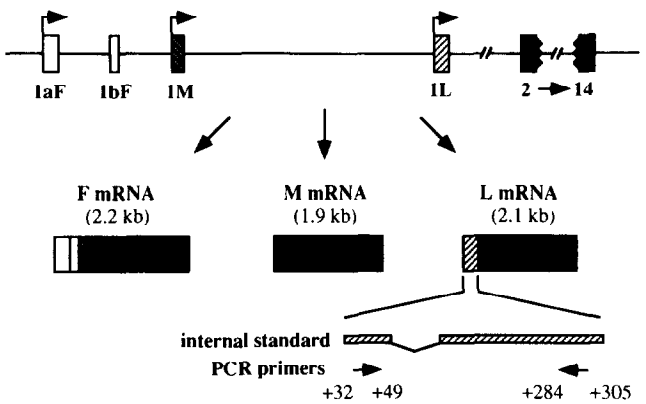

Fig. 1. Structure of the PFK-2/FBPase-2 gene A and of the F-, L- and M-type mRNAs. Boxes represent exons and arrows indicate transcription initiation sites. The lower part of the figure shows the position of the primers and the structure of the internal standard RNA used in the RT-PCR assays.

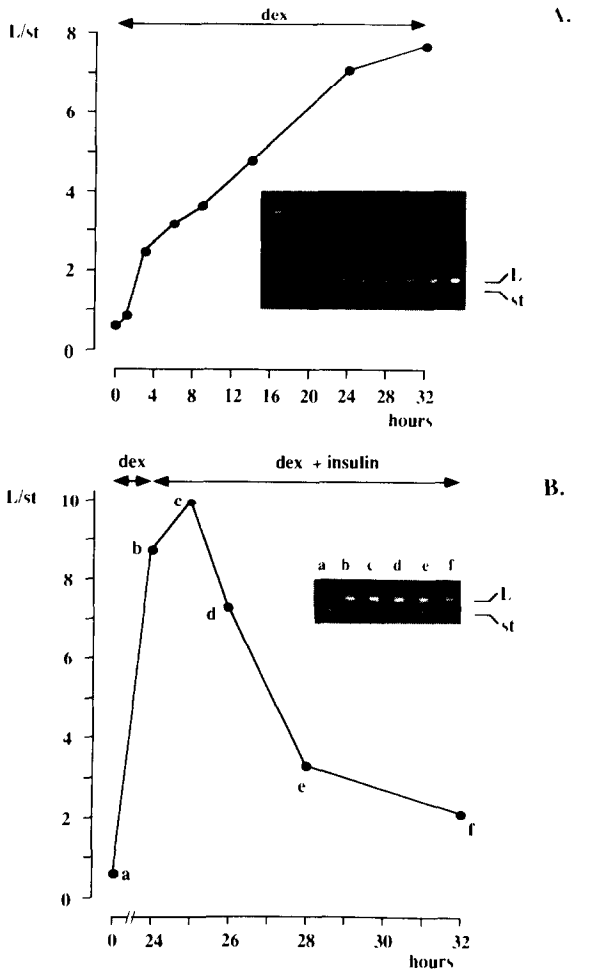

Fig. 2. Insulin reverses the increase in PFK-2/FBPase-2 L mRNA induced by dexamethasone. (A) Effect of 1 μM dexamethasone on L mRNA abundance as a function of time. (B) Reversal by 10 nM insulin of the glucocorticoid-induced stimulation. The abundance of the PFK-2/FBPase-2 L mRNA is expressed as the ratio of the cDNAs amplified by PCR from the RNA corresponding to exon 1 L (L) and to the internal standard (st). Fixed amounts of internal standard RNA (0.04 pg) and of test RNA (500 ng) were used in all reactions. The insets show photographs of the agarose gels stained with ethidium bromide. The L/st ratio was calculated from the radioactivity incorporated in the PCR products.

3. Results and discussion

Because the mRNAs from gene A are rare and the L specific region is limited to exon 1L it is difficult to quantitate L mRNA concentration by conventional hybridization techniques. We have therefore resorted to the sensitive competitive reverse transcription (RT)-polymerase chain reaction (PCR) assay. This technique enables one to quantify the test mRNA, as the amount of internal standard RNA for which equimolar quantities of test and internal standard PCR products are obtained is equal to the amount of test mRNA [11]. To detect variations in L mRNA content, we mixed a fixed amount of RNA extracted from FTO-2B cells with a fixed amount

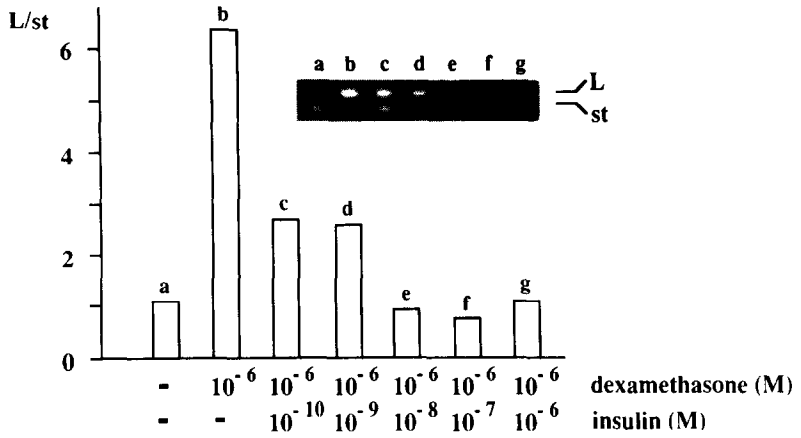

Fig. 3. Dose-dependence of the inhibitory effect of insulin. The cells were incubated for 24 h with hormones, added alone or simultaneously as indicated, and the abundance of PFK-2/FBPase-2 L mRNA was visualised and expressed as in Fig. 2.

of internal standard RNA and measured the ratio of test and internal standard products. Variations of this ratio (L/st) reflect variations in L mRNA concentration in the cells.

To assess the validity of this method, we first measured the variation in L mRNA as a function of time after incubation of the FTO-2B cells with 1 μM dexamethasone (Fig. 2A). This showed an increase in L mRNA concentration after 1 h, followed by a steady rise until a maximum was reached after 32 h of incubation with the hormone. The maximal stimulation was about ten-fold, as determined in separate RT-PCR experiments where the cellular L mRNA was titrated against the internal standard (not shown). A dose–response curve showed that maximal stimulation was obtained at a dexamethasone concentration of 10 nM (not shown). We conclude from these experiments that FTO-2B cells express the PFK-2/FBPase-2 L mRNA and that the concentration of this mRNA increases upon glucocorticoid treatment, as expected.

To test the effect of insulin, cells were incubated for 2–24 h with a saturating concentration (10 nM). No reproducible effect of insulin alone on L mRNA could be detected (not shown). However, insulin exerted a striking inhibitory effect on the dexamethasone stimulation. Fig. 2B shows a representative experiment in which cells were treated with 1 μM dexametha-sone for 24 h. After this period, 10 nM insulin was added to the medium containing dexamethasone and the cells were collected at several time-points. One sees that L mRNA concentration decreased after 2 h to reach, after 8 h, a value close to the unstimulated level. This inhibitory effect was also observed in cells grown in medium depleted of serum starting 24 h prior to adding dexamethasone (not shown).

The effect of insulin was dose-dependent. Cells were incubated for 24 h with 1 μM dexamethasone alone or with dexamethasone and different concentrations of insulin. Fig. 3 shows that glucocorticoid stimulation was inhibited at 0.1 nM insulin and that maximal inhibition was obtained at 10 nM. This effect of insulin is therefore observed in the physiological concentration range.

To determine whether insulin affected the degradation rate of the L mRNA, we stimulated the cells for 24 h with 1 μM dexamethasone, and then added the transcription inhibitor actinomycin D (5 μg/ml) with or without 10 nM insulin (Fig. 4). The cells were harvested at several time-points to measure L mRNA concentration. Insulin did not influence the degradation rate of L mRNA under conditions in which gene transcription was blocked. This experiment did not exclude the possibility that insulin induces, via an actinomycin D-sensitive step, the synthesis of a short-lived protein that accelerates L mRNA degradation. We therefore tested whether the inhibitory effect of insulin required ongoing protein synthesis.

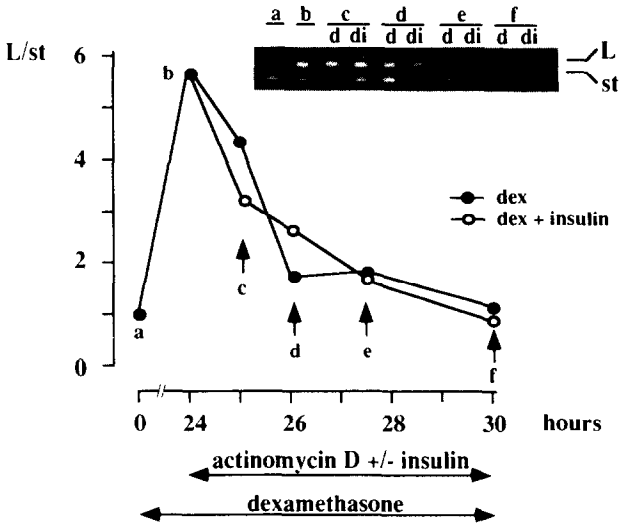

Fig. 4. Insulin does not influence L mRNA degradation. The cells were treated with 1 μM dexamethasone, 10 nM insulin, and actinomycin D (5 μg/ml) as indicated. 'd' and 'di' point to dexamethasone and dexamethasone + insulin-treated samples, respectively. L mRNA abundance is expressed as in Fig. 2.

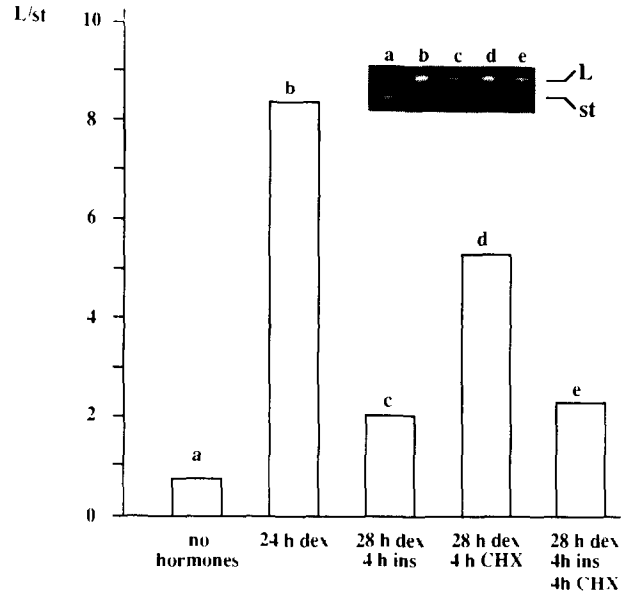

Fig. 5. Inhibition by insulin of the glucocorticoid-induced increase in L mRNA does not require ongoing protein synthesis. Cells were treated with hormones and cycloheximide (CHX) as explained in the text. L mRNA abundance is expressed as in Fig. 2.

FTO-2B cells were incubated for 24 h with 1 μM dexamethasone following which 10 nM insulin was added in the presence of the protein synthesis inhibitor cycloheximide. Cycloheximide (10 μM) was added 15 min before insulin and the cells collected 4 h after addition of insulin. As shown in Fig. 5, the insulin-induced inhibition was independent of protein synthesis. We conclude from these experiments that insulin does not affect the degradation rate of L mRNA and therefore exerts its effect at the level of transcription.

There are two modes of action of insulin on gene transcription [1]. The response of some genes, such as those encoding glucokinase and phosphoenolpyruvate carboxykinase, is fast and is independent of protein synthesis and of the presence of glucose [12,13]. The effect on other genes, such as those encoding aldolase B and pyruvate kinase, is slow in onset and requires glucose and ongoing protein synthesis [14]. Data in Figs. 2 and 5 show that the inhibition exerted by insulin on glucocorticoid induction of the L mRNA was fast and independent of protein synthesis. We therefore tested whether glucose was required for this effect of insulin. The cells were grown for 24 h without hormones, with dexamethasone alone, or with dexamethasone plus insulin, in medium containing glucose (2 g/l) or not. As shown in Fig. 6 glucose was not required for the inhibitory effect of insulin.

In conclusion, we demonstrate here that rat hepatoma FTO-2B cells express the PFK-2/FBPase-2 L mRNA and that glucocorticoids increase its concentration as expected from the stimulation of transcription described in rat liver [7] and Fao hepatoma cells [8]. We also show that insulin can prevent and reverse this effect. Glucocorticoids exert a net gluconeogenic effect by stimulating the activity of transaminases, phosphoenolpyruvate carboxykinase and glucose-6-phosphatase [15]. As the physiological effect of insulin in liver is to stimulate glycolysis, it is not surprising that insulin opposes the effect of glucocorticoids as we show here. Thus, the pattern of hormonal regulation of PFK-2/FBPase-2 L mRNA resembles that of the gluconeogenic enzymes. The increase of PFK-2/FBPase-2 mRNA content in the liver of rats treated with glucocorticoids correlates with an increase in fructose 2,6-bisphosphate content [7]. This in turn could result in an increase in substrate cycling that may lead to amplification of gluconeogenesis. Insulin alone, i.e. in the absence of glucocorticoids, stimulates transcription of the L type PFK-2/FBPase-2 mRNA two- to four-fold [8]. We did not observe such an effect, probably because RT-PCR does not detect low levels of stimulation. The fact that insulin stimulates, even weakly, transcription from the PFK-2/FBPase-2 L promoter when used alone, and inhibits transcription when combined with a glucocorticoid, shows that insulin can exert opposite effects on the same gene depending on the hormonal status. A similar phenomenon has been described for the gene encoding tyrosine aminotransferase [16,17].

Acknowledgements: We thank Dr. G. Schütz (Heidelberg) for generously providing the FTO-2B cells, Louis Hue for continued interest and Th. Lambert for secretarial assistance. This work was supported in part by the Fund for Medical Scientific Research (Belgium) and by the Belgian Programme on Interuniversity Poles of Attraction initiated by the Belgian State Prime Minister's Office of Science Policy Programming

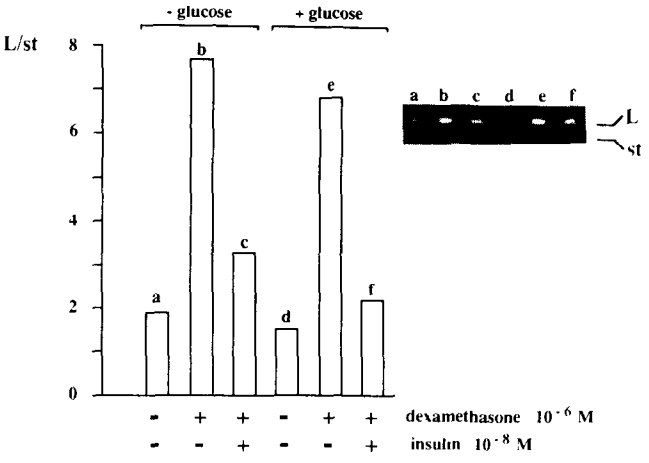

Fig. 6. Inhibition by insulin of the glucocorticoid-induced increase in L mRNA does not depend on extracellular glucose. Cells were treated for 24 h with dexamethasone alone or with a mixture of dexamethasone and insulin in the absence or presence of glucose (2 g/l), as indicated. L mRNA abundance is expressed as in Fig. 2.

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