Protein synthesis is involved in the modulation of the level of the phosphoenolpyruvate carboxykinase mRNA by changes in cell volume in isolated rat hepatocytes

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Abstract The mechanism of action of hydration state was studied on phosphoenolpyruvate carboxykinase (PCK) gene expression in isolated rat hepatocytes. Hypoosmolarity decreased the level of the PCK mRNA after a lag period of about 60 min. The decreasing effect of hypoosmolarity was totally blocked by inhibitors of both protein synthesis and gene transcription. Moreover, hypoosmolarity specifically increased the synthesis of a 45 000 M_r protein, which decreased in the presence of inhibitors of transcription. A close relationship between the synthesis of the 45 000 M_r protein and the decrease in the PCK mRNA level was observed, suggesting that this protein might potentially be involved in the regulation of the level of the PCK mRNA by cell swelling.

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Key words: Hydration state; Hepatocyte; Phosphoenolpyruvate carboxykinase mRNA; Protein synthesis

1. Introduction

In 1994, Newsome et al. [1] reported that cell swelling decreased and cell shrinkage increased the level of the phosphoenolpyruvate carboxykinase (EC 4.1.1.32; PCK) mRNA in perfused rat liver and in H4IIE hepatoma cells. Although such modulations were recently confirmed [2] and extended to isolated rat hepatocytes [3], there is no information available concerning the mechanism by which changes in cell volume may modulate the level of the PCK mRNA. Searching to identify this mechanism, we observed that the decreasing effect of cell swelling required a lag period (more than 60 min) using isolated rat hepatocytes. Interestingly, cell swelling has been reported to increase the level of the β-actin mRNA through a transcriptional mechanism without any lag period [4]. Since cell shrinkage has been reported to decrease hepatic protein synthesis [5], the lag period observed in the effect of cell swelling on the PCK mRNA might correspond to the time required for the synthesis of a protein involved in this effect.

This work was therefore undertaken to test this possibility. The obtained results clearly demonstrate that protein synthesis is involved in the modulation of the level of the PCK mRNA by changes in cell volume. The results also demonstrate that cell swelling specifically increased the synthesis of a 45 000 M_r protein.

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2. Materials and methods

2.1. Materials

α-Amanitin, cordycepin, cycloheximide, 5,6-dichloro-1β-D-ribofuranosylbenzimidazole (DRB), emetin and puromycin were purchased from Sigma. Actinomycin D was from Calbiochem and guanidinium thiocyanate was from Fluka (Basel, Switzerland). L-[^{35}S]Methionine, [α-^{32}P]dCTP (specific activity 3000 Ci/mmol) and Hyperfilm MP were from Amersham. The probes used were the full-length chicken β-actin cDNA provided by Dr D.W. Cleveland [6], an insert of rat PCK cDNA provided by Dr. R.W. Hanson [7] and an insert of rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (EC 1.2.1.12) used as internal control, provided by Dr. J.M. Blanchard [8]. Other biochemical reagents were of analytical grade.

2.2. Isolated liver cells

Hepatocytes were prepared as described previously [9] from 24 h starved male Wistar rats (200–220 g). The cells (usually 50–70 mg wet weight/ml) were shaken (165 strokes/min) in stoppered scintillation vials at 37°C for the indicated times. The standard incubation medium was a Krebs-Henseleit bicarbonate buffer at pH 7.4. Hypo- and hyperosmotic media were obtained by decreasing (−50 mM) or increasing (+35 mM), respectively, the NaCl concentration of the buffer. All media were in equilibrium with a gas phase of O_2/CO_2 (19:1). For RNA determination, the cells were spun down rapidly at +4°C (2000× *g*, 30 s) at the end of the incubation period and the pellets stored at −80°C.

2.3. Extraction and analysis of cellular RNA

Isolation of total RNA was performed by a guanidinium thiocyanate procedure [10]. RNA was separated on 1.5% agarose/formaldehyde gels and transferred to nylon membrane for Northern hybridization. Membranes were hybridized using random oligonucleotide-primed ^{32}P-labelled insert as described [11]. Filters were washed and exposed to Hyperfilm at −80°C using intensifying screens. Relative densities of the hybridization signals were quantified by scanning the films with a Shimadzu densitometer. To correct for differences in RNA loading, all the results were expressed as the ratio of the scanned values for β-actin or PCK mRNAs versus those for GAPDH mRNA (relative level).

2.4. Determination of protein synthesis

L-[^{35}S]Methionine was added to the incubation medium at 20 nM (1000 Ci/mmol). At the end of incubation, hepatocytes were diluted in an equal volume of ice-cold 10 mM HEPES buffer, pH 7.4, and spun down rapidly (1 min at 500× *g*). The cell pellet was immediately resuspended in 1 ml of cold buffer and homogenized with a Dounce homogenizer (30 strokes). The cell extract was clarified by centrifugation (10 000× *g*, 10 min). 10 μl supernatant was subjected to SDS-PAGE in 8% (w/v) polyacrylamide gel (minigel or 18×24 cm gel). After electrophoresis, the gel was stained with Coomassie blue and exposed to Hyperfilm for autoradiography. Standard molecular weight markers (Sigma) were run in parallel to calculate relative molecular masses.

2.5. Expression of the results

The results are expressed as means ± S.E.M. for the observations on the indicated number (*n*) of different cell preparations. Statistical significance of differences was calculated with Student's *t*-test for paired data.

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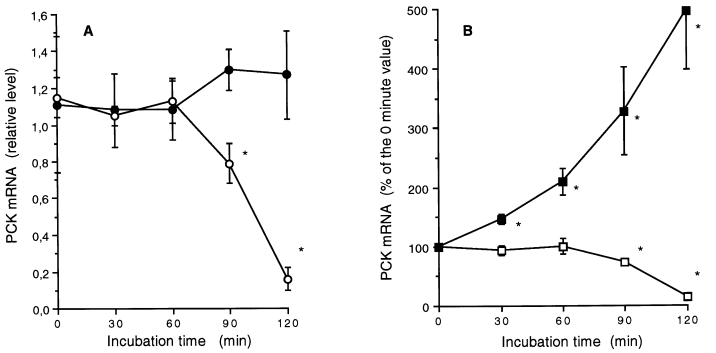

Fig. 1. Time course study of the effect of hypoosmolarity on the level of PCK and β-actin mRNAs. Hepatocytes were incubated with 5 mM glucose in hypo- or hyperosmotic media for different periods of time. Total RNA was extracted and 20 μg aliquots analyzed by Northern blot. They were probed successively with the PCK, the β-actin and the GAPDH cDNAs. The results are means ± S.E.M. for six different cell preparations. *, Significantly different from the 0 min values. A: Influence of hypoosmolarity on the level of the PCK mRNA. (○), hypo; (●), hyper. B: Comparison of the effect of hypoosmolarity on the level of the PCK and β-actin mRNAs. (□) PCK; (■) β-actin.

3. Results and discussion

3.1. Inhibitors of protein synthesis totally blocked the decreasing effect of hypoosmolarity on the PCK mRNA level

Hepatocytes isolated from 24 h fasted rats were incubated for different periods of time in hypo- and hyperosmotic media. As shown in Fig. 1A, hypoosmolarity decreased the level of the PCK mRNA and this effect appeared significant 90 min after the beginning of the incubation. However, the hypoosmotic medium was obtained by decreasing the NaCl concentration. Thus, an effect of the change in the NaCl concentration could not be excluded to explain the observed decrease in the level of the PCK mRNA. The effect of raffinose addition was therefore tested on the level of the PCK mRNA in hypoosmotic condition. Hepatocytes were incubated for 2 h in hypoosmotic condition with or without raffinose. Addition of 80 mM raffinose inhibited the decreasing effect of hypoosmolarity on the level of the PCK mRNA (100%, hyper; 38.5 ± 5.8%*, hypo; 77.0 ± 6.3%*, hypo+raffinose; $n = 5$; $P < 0.05$). These results (i) confirmed that cell swelling was responsible for the decreasing effect of hypoosmolarity on the level of the PCK mRNA and (ii) demonstrated that the effect of cell swelling required a lag period greater than 60 min. The notion of a lag period in the effect of hypoosmolarity on the regulation of the expression of the PCK gene was also reinforced by the fact that, in the same experiments, the effect of hypoosmolarity on the level of the β-actin mRNA appeared in the first minutes of the incubation period (Fig. 1B). Then, we tested the influence of inhibitors of protein synthesis on the decreasing effect of hypoosmolarity on the level of the PCK mRNA. Hepatocytes were therefore incubated in hypoosmotic medium with or without emetin (a known inhibitor of protein synthesis) [12]. As shown in Fig. 2, addition of 0.1 mM emetin blocked the decreasing effect of hypoosmolarity on the PCK mRNA level and the obtained mRNA level was not significantly different from that measured in hyperosmotic condition (0.36 ± 0.07, hypo; 1.13 ± 0.09*, hypo+emetin; 1.03 ± 0.10*, hyper; $n = 5$; $P < 0.05$). The same inhibitory effect was obtained using cycloheximide or puromycin, two other known inhibitors of protein synthesis (not shown). Moreover, emetin was without any significant effect on the level on the PCK mRNA in hepatocytes incubated in hyperosmotic conditions (1.03 ± 0.10, hyper; 1.04 ± 0.03, hyper ± emetin; $n = 5$) (see also Fig. 2). These results demonstrate that protein synthesis was involved

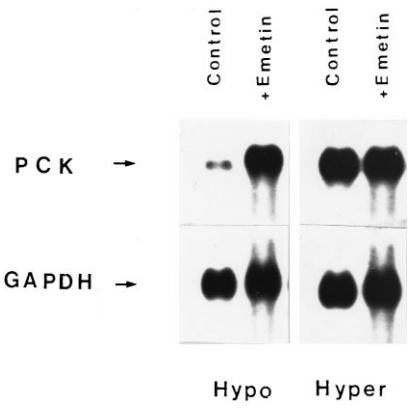

Fig. 2. Influence of emetin on the effect of hypoosmolarity on the PCK mRNA level. Hepatocytes were incubated for 2 h in the presence of 5 mM glucose in hypo- or hyperoosmotic condition with or without emetin (0.1 mM). Total RNA was extracted and 20 μg aliquots analyzed by Northern blot. They were probed successively with the PCK and the GAPDH cDNAs.

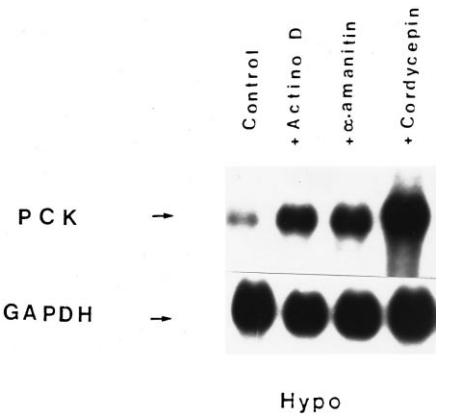

Fig. 3. Influence of inhibitors of transcription on the effect of hypoosmolarity on the PCK mRNA level. Hepatocytes were incubated for 2 h in hypoosmotic condition in the presence of 5 mM glucose with or without actinomycin D (0.5 μg/ml), α-amanitin (0.5 μg/ml) or cordycepin (50 μg/ml). Total RNA was extracted and 20 μg aliquots analyzed by Northern blot. They were probed successively with the PCK and the GAPDH cDNAs.

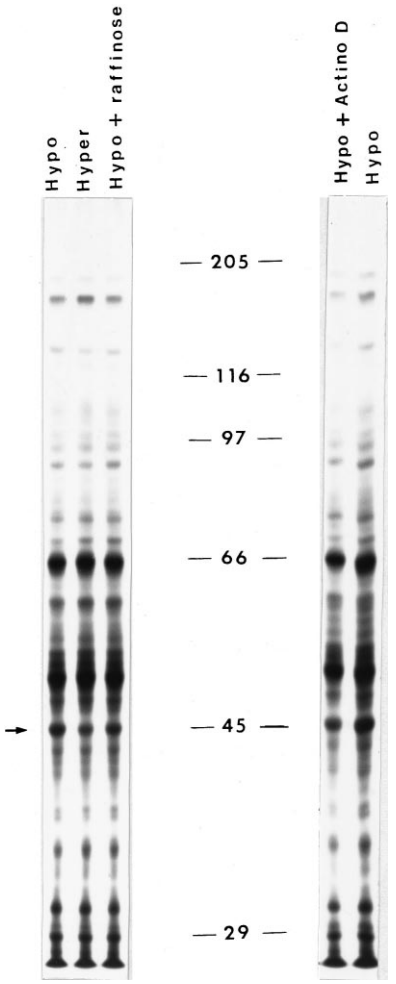

Fig. 4. Influence of hypoosmolarity on protein synthesis. Hepatocytes were incubated for 2 h in the presence of 5 mM glucose with L-[^{35}S]methionine (20 nM; 1000 Ci/mmol). Total homogenates were subjected SDS-PAGE in 8% (w/v) polyacrylamide gel (18×24 cm) for autoradiography as described in Section 2. Left: Influence of the addition of raffinose on the effect of hypoosmolarity. Hepatocytes were incubated in hyperosmotic condition or in hypoosmotic condition with or without raffinose (80 mM). Right: Influence of actinomycin D on the effect of hypoosmolarity. Hepatocytes were incubated in hypooosmotic condition with or without actinomycin D (0.5 μg/ml).

in the decreasing effect of hypoosmolarity on the PCK mRNA level.

3.2. *Inhibitors of gene transcription totally blocked the decreasing effect of hypoosmolarity on the PCK mRNA level*

To specify the modalities of action of hypoosmolarity, we tested the influence of inhibitors of gene transcription. Hepatocytes were incubated for 2 h in hypoosmotic medium with or without actinomycin D, a known inhibitor of gene transcription. Fig. 3 shows that actinomycin D totally blocked the decreasing effect of hypoosmolarity on the level of the PCK mRNA (0.22 ± 0.04, hypo; 1.31 ± 0.11*, hypo+actinomycin D; 1.16 ± 0.21*, hyper; $n = 5$; $P < 0.05$). The same inhibitory effect was obtained using α-amanitin or cordycepin, two other known inhibitors of transcription (Fig. 3). This was also observed when using DRB, another inhibitor of transcription (not shown). This demonstrates that the activation of gene transcription was involved in the decreasing effect of hypoosmolarity on the level of the PCK mRNA. Taken together, all these results clearly demonstrate that an increase in both gene transcription and protein synthesis was involved in the effect of hypoosmolarity. This strongly suggests that (i) protein(s) might be rapidly synthesized under the influence of hypoosmolarity and (ii) this (these) protein(s) might be responsible for the observed decrease in the level of the PCK mRNA.

3.3. *Hypoosmolarity increased the synthesis of a 45 000 M_r protein*

To verify that changes in cell volume rapidly modulate protein synthesis, hepatocytes were incubated for 2 h in hypo- and hyperosmotic media with L-[^{35}S]methionine. The obtained results show that the incorporation of L-[^{35}S]methionine increased in hepatocytes incubated in hypoosmotic medium, as compared to hyperosmotic medium (100%, hyper; $132.9 \pm 11.1\%$*, hypo; $n = 6$; $P < 0.05$). Although the observed increase was smaller than that reported by Stoll et al. using L-[^{3}H]leucine as marker [5], these results confirm that protein synthesis was higher in hypoosmotic condition than in hyperosmotic condition. Then, we tried to identify specific protein(s) whose synthesis particularly increased in hypoosmotic condition. Total homogenates prepared from the preceding experiments were therefore subjected to SDS-PAGE and autoradiographed. As shown in Fig. 4, one major band corresponding to a 45 000 M_r protein specifically increased in hepatocytes incubated in hypoosmotic condition. The same results were obtained in five independent experiments. Fig. 4 also shows that the addition of raffinose decreased the stimulatory effect of hypoosmolarity on the synthesis of the 45 000 M_r protein. This demonstrates that the synthesis of the 45 000 M_r protein was modulated by a change in cell volume. Moreover, Fig. 4 also shows that the synthesis of the 45 000 M_r protein decreased in the presence of actinomycin D. The same result was obtained using α-amanitin (not shown). This demonstrated that a transcriptional mechanism was involved. However, this does not necessarily imply that the transcription of the gene corresponding to the 45 000 M_r protein is the regulatory step. Then, we tried to demonstrate that the 45 000 M_r protein might be involved in the regulation of the level of

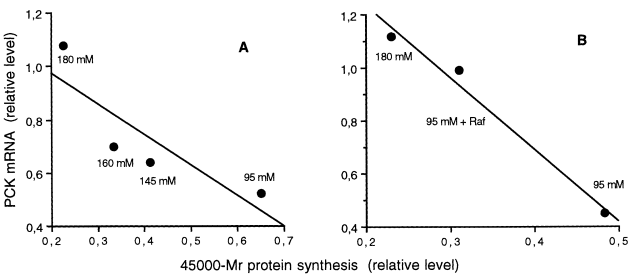

Fig. 5. Correlation between the level of the PCK mRNA and the level of the synthesis of the 45 000 M_r protein. Hepatocytes were incubated for 2 h in different osmotic conditions with 5 mM glucose in the presence (protein measurement) or in the absence of L-[^{35}S]methionine (20 nM; 1000 Ci/ mmol) (mRNA measurement). Total homogenates were subjected to SDS-PAGE and autoradiographed, and the signal corresponding to the 45 000 M_r protein scanned. Total RNA was extracted and 20 μg aliquots analyzed by Northern blot. They were probed successively with the PCK and the GAPDH cDNAs. A: Influence of anisotonicity. Correlation coefficient = 0.86. B: Influence of the addition of raffinose (80 mM) on the effect of hypoosmolarity. Correlation coefficient = 0.99.

the PCK mRNA. Hepatocytes were incubated for 2 h in different osmotic conditions in the presence of L-[^{35}S]methionine. The level of both the PCK mRNA and the 45 000 M_r protein synthesis was measured. As shown in Fig. 5A, the 45 000 M_r protein synthesis increased concomitantly with the decrease in the osmolarity of the extracellular medium, and a close relationship may be established between the level of the PCK mRNA and the synthesis of the 45 000 M_r protein. Similar results were obtained in two independent experiments. To specify that the relationship involved changes in cell volume, hepatocytes were incubated for 2 h in hyperosmotic condition and in hypoosmotic condition with or without raffinose (80 mM). As shown in Fig. 5B, such a relationship may also be established in these experimental conditions. Taken together, these results strongly suggest that the 45 000 M_r protein might be involved in the modulation of the level of the PCK mRNA by changes in cell volume. Interestingly, two proteins were recently shown to regulate the PCK mRNA degradation: a 100 000 M_r protein [13] and a 24 500 M_r protein [14]. This suggests that the 45 000 M_r protein might be different from these two identified proteins.

In conclusion, the data reported here demonstrate that hypoosmolarity decreased and hyperosmolarity increased the PCK mRNA level indirectly through an inverse modulation of protein synthesis. This is, to our knowledge, the first report that modulations of hydration state required protein synthesis to regulate gene expression in the liver. Moreover, the data reported here demonstrate that changes in cell volume modulate the synthesis of a 45 000 M_r protein. However, the involvement of the 45 000 M_r protein in the modulation of the PCK mRNA level remains to be firmly established.

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