AMP-activated protein kinase counteracted the inhibitory effect of glucose on the phosphoenolpyruvate carboxykinase gene expression in rat hepatocytes

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Abstract The effect of AMP-activated protein kinase (AMPK) in the regulation of the phosphoenolpyruvate carboxykinase (PEPCK) gene expression was studied in isolated rat hepatocytes. Activation of AMPK by AICAR counteracted the inhibitory effect of glucose on the PEPCK gene expression, both at the mRNA and the transcriptional levels. It is proposed that a target for AMPK is involved in the inhibitory effect of glucose on PEPCK gene transcription. **© 2000 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.**

Key words: AMP kinase; Phosphoenolpyruvate carboxykinase; Glucose; AICAR; Hepatocyte

1. Introduction

The AMP-activated protein kinase (AMPK) is the mammalian homolog of the yeast Snf1 protein kinase which is involved in derepression of glucose-repressed genes (for review, see [1]). Recently, AMPK has been shown to inhibit the transcriptional activation by glucose of three positively regulated genes, namely L-type pyruvate kinase [2], spot 14 [2] and fatty acid synthase [2,3] genes in rat hepatocytes. In opposite, glucose has been reported to negatively regulate the expression of the phosphoenolpyruvate carboxykinase (PEPCK) gene at a transcriptional level [4,5]. It may therefore be speculated that, by analogy to its effect on the genes positively regulated by glucose, AMPK might also oppose to the inhibitory effect of glucose on the PEPCK gene expression. This work was therefore undertaken to specify whether AMPK activation may (i) regulate the expression of the PEPCK gene and (ii) inhibit the transcriptional effect of glucose on the gene in rat hepatocytes. To this end, 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR), a specific activator of AMPK activity [6], was used in isolated hepatocytes. The obtained results demonstrate that AMPK is involved in the regulation by glucose of the PEPCK gene expression.

2. Materials and methods

2.1. Materials

AICAR and α-amanitin were purchased from Sigma. Guanidium thiocyanate was from Fluka (Basel, Switzerland). [α-^{32}P]dCTP (specific activity 3000 Ci/mmol), [α-^{32}P]dUTP (specific activity 800 Ci/mmol), [γ-^{32}P]ATP (specific activity 500 Ci/mmol), Hybond-N membranes, multiprime DNA labelling kit and Hyperfilm MP were from Amersham.

The probes and plasmids used were an insert of rat PEPCK cDNA provided by Dr. R.W. Hanson [7], an insert of rat glyceraldehyde-3-phosphate dehydrogenase (GraP-DH) cDNA provided by Dr. J.M. Blanchard [8] and a plasmid containing the 18S rRNA insert provided by Dr. A. Mazur (INRA, Clermont Ferrand, France).

2.2. Incubation of hepatocytes

Hepatocytes were prepared as described previously [9] from 24 h starved male Wistar rats (180–220 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. For RNA determination, the cells were spun down rapidly at 4°C ($2000\times g$, 30 s) at the end of the incubation period and the pellets stored at −80°C.

2.3. Extraction and analysis of RNA

Isolation of total RNA was performed by a guanidium 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] and they were washed and exposed to Hyperfilm at −80°C, using intensifying screens. Relative densities of the hybridization signals were quantified by densitometric scanning. To correct for differences in RNA loading, all the results were expressed as the ratio of the scanned values for PEPCK mRNA versus those for GraP-DH mRNA (relative level).

2.4. Nuclear run-on transcription assay

The preparation of nuclei and the RNA-polymerase elongation reaction were performed essentially as described [12]. For transcription, 8×10^6 nuclei were incubated for 30 min at 28°C in 200 μl 15% glycerol, 50 mM HEPES pH 8.0, 150 mM KCl, 1 mM dithiothreitol, 2.5 mM magnesium acetate, 1 mM $MnCl_2$, 0.5 mM EDTA, 4 mM creatine phosphate, 15 U/ml creatine kinase, 1 mM spermidine, 0.5 mM each of ATP, CTP and GTP, and 500 U/ml RNasin, in the presence of 100 μCi of [α-^{32}P]dUTP. DNase I (40 U) was added and the samples were incubated at 37°C for 15 min. The labelled RNAs were extracted as described [10]. Denatured plasmids (5 μg) were spotted onto cellulose using a slot blot apparatus. Each DNA bearing filters was prehybridized then hybridized with labelled RNA (5×10^6 cpm) at 42°C for 3 days. Hybridization was carried out with three separate plasmids: the PEPCK cDNA, a non-recombinant pUC plasmid as a control for non-specific binding of labelled RNA, and the 18S rRNA probe as internal control. Filters were washed and exposed to Hyperfilm at ± 80°C with intensifying screens. The relative amount of labelled transcripts hybridized to plasmids was determined by densitometric scanning of autoradiograms. The values obtained for the PEPCK signal were corrected using the 18S values.

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2.5. Measurement of AMPK activity

Cytosolic extracts were prepared as described by Forest et al. [3], including the PEG step. Aliquots (5 μl) were used to measure the AMPK activity by the SAMS peptide phosphorylation assay in the presence of 200 μM 5′-AMP, as described by Davies et al. [13] (25 μl final assay volume). AMP kinase activity is expressed as nmol of phosphate incorporated into SAMS peptide/min/mg protein (U/mg protein).

2.6. Expression of results

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 by the Student's t-test for paired data.

3. Results and discussion

3.1. AICAR maintained the PEPCK mRNA level

Hepatocytes were incubated for 120 min in the presence of 5 mM glucose with or without 500 μM AICAR. AICAR addition induced a significant increase in the PEPCK mRNA level: 0.57 ± 0.08, control; 1.73 ± 0.38, +500 μM AICAR; $n = 7$; $P < 0.05$. The half-maximum effect was observed at about 150 μM AICAR (Fig. 1). In order to specify the modalities of action of AICAR, we studied the time-course of the effect of AICAR. Hepatocytes were incubated with or without 500 μM AICAR for various periods of time, as indicated, and the PEPCK mRNA level was measured. The obtained results showed that, firstly, the PEPCK mRNA level decreased after the first hour of the incubation period in control cells and, secondly, AICAR addition maintained the PEPCK mRNA level to its initial value throughout the experiment (Fig. 2). This demonstrated that the main effect of AICAR was to maintain the PEPCK mRNA level to its initial value. Since AICAR is known to induce an increase in the AMPK activity, this suggested that the effect on the PEPCK mRNA might be a consequence of AMPK activation. We therefore studied the possible relationship between AMPK activation and PEPCK gene expression.

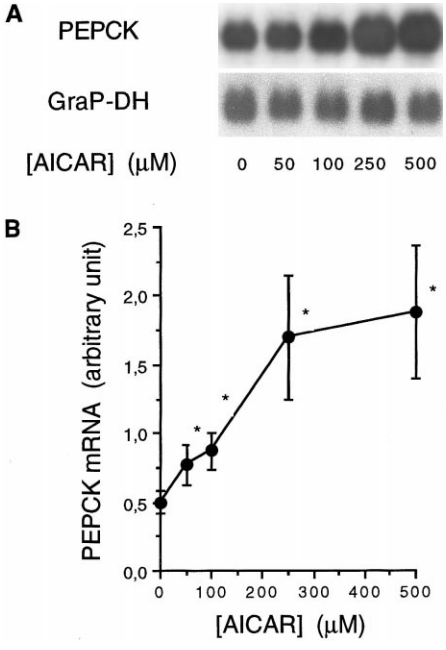

Fig. 1. Dose-response of the effect of AICAR on the PEPCK mRNA level. Hepatocytes were incubated for 120 min with 5 mM glucose in the presence of various concentrations of AICAR, as indicated. Total RNA was extracted and 20 μg aliquots analyzed by Northern blot. They were probed successively with the PEPCK and the GraP-DH cDNAs. A: Representative autoradiogram. B: Scanned data of autoradiograms. Values are expressed as means ± S.E.M. for five different cell preparations. *: significantly different ($P < 0.05$) from the control value.

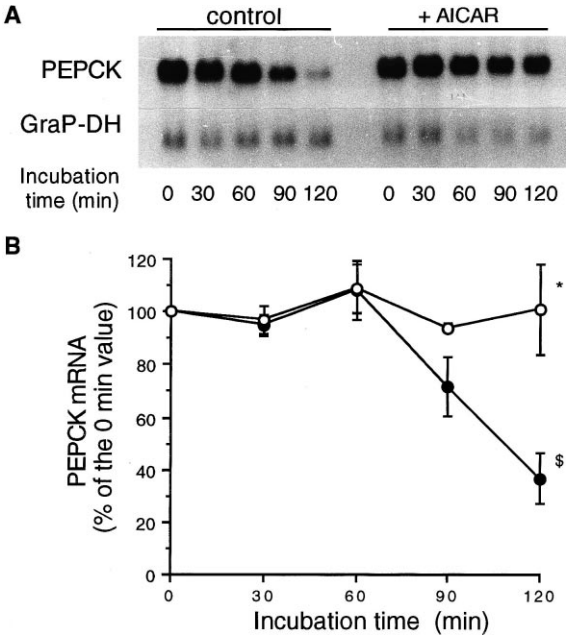

Fig. 2. Time-course study of the effect of AICAR on the PEPCK mRNA level. Hepatocytes were incubated with 5 mM glucose in the absence (●) or in the presence of 500 μM AICAR (○) for various periods of time, as indicated. Total RNA was extracted and 20 μg aliquots analyzed by Northern blot. They were probed successively with the PEPCK and the GraP-DH cDNAs. A: Representative autoradiogram. B: Scanned data of autoradiograms. Values are expressed as means ± S.E.M. for five different cell preparations. *: significantly different ($P < 0.05$) from the corresponding control value. $: significantly different ($P < 0.05$) from the 0 min value.

3.2. AMPK activation is responsible for the effect of AICAR on the PEPCK gene expression

We first studied the time-course of the kinase activation under the influence of AICAR. Hepatocytes were incubated in the presence of 5 mM glucose with or without 500 μM AICAR for various periods of time, as indicated. As shown in Fig. 3, AICAR activated AMPK in the first minutes of the incubation period and this effect was maximal between 10 and 15 min. To study the possible relationship between AMPK activity and PEPCK mRNA level, hepatocytes were therefore incubated in the presence of 5 mM glucose with various concentrations of AICAR, and AMPK activity and PEPCK mRNA level were measured at 15 min and 120 min, respectively. As shown in Fig. 4, AICAR dose-dependently increased the PEPCK mRNA level and a close relationship may be established between PEPCK mRNA level and APMK activity. This strongly suggested that the observed effect of AICAR on the PEPCK mRNA level might be a consequence of AMPK activation. In order to specify this point, AMPK activation was induced by incubating the cells without O_2. As expected, hypoxia induced an increase in the AMPK activity as measured after 15 min of incubation

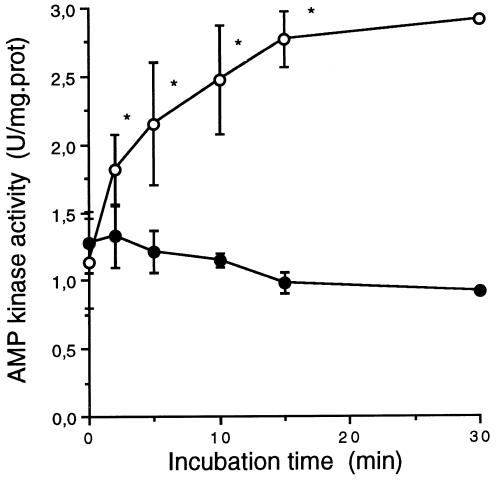

Fig. 3. Time-course study of the effect of AICAR on the AMP kinase activity. Hepatocytes were incubated with 5 mM glucose in the absence (●) or in the presence of 500 μM AICAR (○) for various periods of time, as indicated. Cells were collected and AMPK assay was performed as described. Values are expressed as means ± S.E.M. for four different cell preparations, except for 30 min ($n=2$). *: significantly different ($P<0.05$) from the 0 min value.

(1.43 ± 0.30 U/mg protein, control; 2.15 ± 0.36* U/mg protein, no O_2) and this was associated with an increase in the PEPCK mRNA level at the end of the experiment (i.e. 120 min) (100%, control; 242.9 ± 34.8%*, no O_2; $n=3$; $P<0.05$). This demonstrated that AMPK activation was responsible for the effect of AICAR on the PEPCK mRNA level.

3.3. AMPK activation acts at a transcriptional level

In order to specify whether a transcriptional mechanism was involved, run-on transcription assays were performed. Hepatocytes were incubated for 100 min in the presence of 5 mM glucose with or without 250 μM AICAR. As shown in Fig. 5 for a representative experiment, AICAR addition induced an increase in the rate of transcription of the PEPCK gene (1, control; 1.76 ± 0.18, +AICAR; $n=4$; $P<0.05$). Moreover, this figure also shows that such an increase (1, control; 2.04, without O_2; $n=2$) was observed in cells incubated without O_2, a condition known to activate AMPK (vide supra). This demonstrated that AMPK activation acts at a transcriptional level. Glucose has been reported to decrease the rate of transcription of the PEPCK gene in both isolated [4] and cultured rat hepatocytes [5]. Moreover, we showed that the PEPCK mRNA level decreased in the presence of 5 mM glucose (control cells) but AMPK activation maintained the level at the initial value (Fig. 2). Taken together, these results therefore suggested that AMPK activation might counteract the transcriptional inhibitory effect of glucose on the gene. To specify this point, we compared the rate of transcription of the gene in hepatocytes incubated for 10 and 100 min, respectively, in the presence of 5 mM glucose both with or without 250 μM AICAR. The obtained results showed that glucose induced a decrease of about 50% in the rate of transcription of the PEPCK gene (1, 10 min; 0.45, 100 min) but AICAR addition maintained the rate of transcription of the gene at its initial value (1, 10 min; 1.20, 100 min). Such a result was obtained in two independent experiments confirming that glucose per se decreases PEPCK gene transcription and demonstrating that AMPK activation was able to counteract the transcriptional inhibitory effect of glucose on the PEPCK gene. Although we were not able to measure any change in the PEPCK mRNA stability using 0.5 μg/ml α-amanitin (data not shown), we cannot exclude a post-transcriptional effect of AICAR.

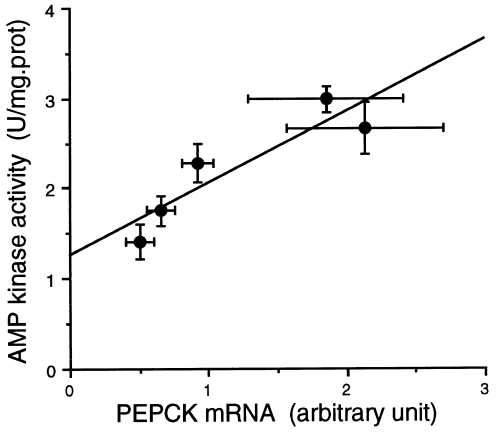

Fig. 4. Correlation between the AMP kinase activity and the PEPCK mRNA level. Hepatocytes were incubated for 15 min (AMPK) and 120 min (mRNA), respectively, with 5 mM glucose in the presence of various concentrations of AICAR (0, 50, 100, 250 or 500 μM). Values are expressed as means ± S.E.M. for four different cell preparations. Correlation coefficient = 0.91.

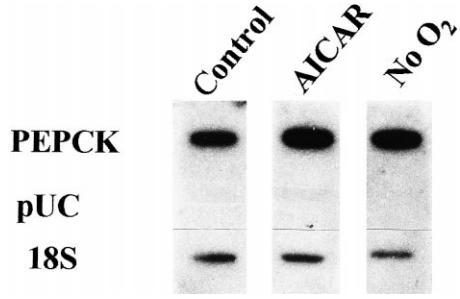

Fig. 5. Influence of AICAR and hypoxia on the rate of transcription of the PEPCK gene. Hepatocytes were incubated for 100 min with 5 mM glucose in the absence (control) or in the presence of 250 μM AICAR, or without O_2. After extraction, nuclei were incubated with [α-^{32}P]UTP, as described. The labelled RNAs were hybridized on to filters containing the PEPCK cDNA, a plasmid pUC for non-specific binding and the 18S rRNA probe as internal control.

In cultured hepatocytes, Leclerc et al. [2] reported that PEPCK mRNA level was not modified by AICAR addition, in contrast with the present results. The very different conditions of hepatocytes culture (no glucose and presence of cAMP, a potent inducer of PEPCK gene expression) may probably explain this discrepancy.

4. Conclusion

In conclusion, the data reported here demonstrated that AMPK activation, firstly, was able to oppose to the inhibitory effect of glucose on the PEPCK gene expression and, secondly, acted at a transcriptional level. Since AMPK has been reported to inhibit the transcriptional activation by glucose of positively regulated genes [2,3], it may therefore be proposed that a target for AMPK was involved in both the

stimulatory and the inhibitory effects of glucose on gene transcription, at least in rat hepatocytes. However, the target involved remains to be identified.

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