The 5′-AMP-activated protein kinase inhibits the transcriptional stimulation by glucose in liver cells, acting through the glucose response complex

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Received 16 June 1998

Abstract 5-Amino-4-imidazolecarboxamide riboside (AICAR) is known to stimulate rat liver 5′-AMP-activated protein kinase (AMPK). AMPK is the mammalian homologue of Snf1p in yeast, involved in derepression of glucose-repressed genes. We used AICAR to test if AMPK could also play a role in the regulation of glucose-dependent genes in mammalian cells. At a concentration which induces phosphorylation-dependent inactivation of HMG-CoA reductase, AICAR blocked glucose activation of three glucose responsive genes, namely L-type pyruvate kinase (L-PK), Spot 14 and fatty acid synthase genes in primary cultured hepatocytes, but was without any action on glucose phosphorylation to glucose 6-phosphate and on expression of PEPCK, albumin and β-actin genes. AICAR was also found to inhibit activation of the L-PK gene promoter by glucose in transiently transfected hepatoma cells. Therefore our results suggest that AMPK is probably involved in the glucose signal pathway regulating gene expression in the liver.

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Key words: 5′-AMP-activated protein kinase; 5-Amino-4-imidazolecarboxamide riboside; Glucose; Pyruvate kinase gene

1. Introduction

The L-type pyruvate kinase gene (L-PK), encoding a key glycolytic enzyme in the liver, is transcriptionally regulated, positively by glucose and insulin and negatively by glucagon and cAMP. Previous studies have demonstrated that this transcriptional regulation by glucose and hormones could be ascribed to the glucose response element (GlRE) which contains two E boxes located from −168 to −144 bp with respect to the cap site [1–3]. Apart from the category of glucose-independent insulin-stimulated genes represented by glucokinase [4], most other insulin-sensitive genes investigated so far seem to require the presence of glucose, for instance the genes for aldolase B [5], phosphofructokinase-2 [6], fatty acid synthase [7], Spot 14 [8], and L-PK [5]. The role of insulin in the activation of these genes seems mainly to stimulate glucokinase synthesis, and to permit glucose phosphorylation [9,10]. Glucose 6-phosphate (G 6-P) is thus the first step of glucose-dependent activation of glucose-responsive genes in hepatocytes, and we have recently suggested that it could act through the pentose phosphate pathway [11]. Although other investigators proposed that G 6-P could be the active metabolite [12], the role of pentose phosphate was recently supported by in vivo studies [13]. In any case, whether the active metabolite is glucose 6-phosphate itself or a pentose phosphate, the signaling pathway from this metabolite to the transcriptional machinery remains unknown. In this paper we propose that the 5′-AMP-activated protein kinase (AMPK) could be involved in this pathway.

Mammalian AMPK is a multisubstrate protein kinase which appears to play a central role in lipid metabolism [14,15]. AMP was first identified to induce phosphorylation and inactivation of HMG-CoA reductase and acetyl-CoA carboxylase, the rate limiting enzymes of cholesterol and fatty acid synthesis, respectively. AMPK is activated by a high AMP/ATP ratio inside the cell which occurs in stressed conditions like heat shock, hypoxia, arsenite treatment and starvation [16]. In blocking anabolic pathways like cholesterol and fatty acids synthesis, it preserves ATP for more vital short-term functions like ion gradients across the plasma membrane. The AMPK is the analogue of Snf1p in yeast and shares with this protein 64% identity at the amino acid sequence level in the catalytic region. The Snf1p protein kinase is essential for derepression of glucose-repressed genes, and permits fermentation of non-glucose sugars [17,18]. In addition, Snf1p seems to be involved in the activation by low levels of glucose of the glucose transporter HXT2 and HXT4 genes, repressed in yeasts cultured in the presence of a high glucose concentration [19]. This leads to the idea that AMPK could also regulate the expression of glucose-regulated genes in mammals. To test this hypothesis, we looked if activation of AMPK by 5-amino-4-imidazolecarboxamide riboside (AICAR) in liver cells blocks the transcription of some glucose-activated genes. Incubation of cells with AICAR causes accumulation of ZMP, an AMP analogue [20] to high level without disturbing the preexisting AMP, ADP and ATP levels. It is actually the most specific method to activate AMPK in intact cells [21]. Our results suggest that AMPK indeed controls the regulation of glucose-responsive genes in mammals as well as in yeast.

2. Materials and methods

2.1. Plasmids

Plasmid constructions were done by standard cloning procedures [22]. The constructs were verified by nucleotide sequencing. The different plasmids constructed (termed −183 PK/CAT, −150 PK/CAT, KSV2/CAT and $(MLP)_4$ −54 PK/CAT) have been previously described [1,9,23].

2.2. Hepatocyte isolation and cell culture conditions

The mhAT3F hepatocyte-like cell line was derived from the tumoral liver of transgenic mice expressing the SV40 large T and small T antigens under the direction of the liver-specific antithrombin III promoter [9,24,25]. Cells were cultured in Ham's F-12-Dulbecco's modified Eagle's medium (v/v) (Life Technologies, Inc.) supplemented with

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PII: S0014-5793(98)00745-5

penicillin, streptomycin, 20 nM insulin, 1 μM triiodothyronine, 1 μM dexamethasone, and 5% (v/v) fetal calf serum. Twenty-four hours before the experiment, cells were cultured in a serum-free medium containing 10 mM lactate and supplemented with the same mixture of hormones as described above.

Hepatocytes were isolated from male Sprague-Dawley rats (180–200 g) or from male B6/CBA mice (18–22 g) by the collagenase perfusion method [1]. Three millions of hepatocytes in suspension were plated on 10-cm dishes in a final volume of 10 ml of 199 medium (Life Technologies Inc.) supplemented with penicillin, streptomycin and 10% (v/v) fetal calf serum. After 12 h of attachment, the medium was removed and replaced by a hormone-supplemented fresh 199 medium without glucose and with 10 mM lactate as a carbon source for 24 h. Then the medium was changed for a fresh 199 medium with different AICAR concentrations in the presence of glucose (25 mM) or lactate (10 mM) for 24 h and total RNA was extracted for Northern blot analysis. For PEPCK mRNA study, 1 mM 8-bromoadenosine 3′,5′-cyclic monophosphate (cAMP) was added in the lactate medium.

2.3. Transfections and CAT assays

Transfection of the mhAT3F cell line was performed by lipofection using *N*-(2,3-dioleoyloxy)propyl-*N*,*N*,*N*-trimethylammonium methylsulfate (DOTAP, Boehringer Mannheim, Mannheim, Germany) according to the manufacturer's instructions. Five μg of the tested constructs (−183 L-PK/CAT, −150 L-PK/CAT or $(MLP)_4$ −54 L-PK/CAT) or 3 μg of the reference KSV2/CAT construct were transfected. The medium containing the liposome-DNA complex was replaced 12 h later with fresh medium containing 10 mM lactate or 20 mM glucose, and AICAR was added at different concentrations. Cells were harvested 36 h after transfection and CAT activity assay was performed as described [1]. The CAT activity was normalized with cellular protein content and with respect to the KSV2/CAT activity as a transfection standard.

2.4. Northern blot analysis

Total RNA was isolated from hepatocytes in primary culture by lysis in guanidinium, followed by phenol extraction [26]. The RNA concentration was determined spectrophotometrically. RNA samples (15 μg) were denatured at 65°C for 15 min and electrophoretically separated on 1% (w/v) agarose-formaldehyde gel, in 1× 3-(*N*-morpholino)propanesulfonic acid (MOPS, Sigma, USA) buffer, transferred to nylon membrane (Hybond-N^+, Amersham), and fixed by heating at 80°C for 1 h. Prehybridization (4 h with 100 μg/ml salmon sperm DNA) and hybridization with L-PK [2], S14 [27], FAS [28], PEPCK and rat serum albumin [24] cDNA probes were carried out as described previously [2]. Each blot was stripped and reprobed with a ribosomal 18S cDNA probe and quantified with PhosphorImager (Molecular Dynamics).

2.5. HMG-CoA reductase assay

The HMG-CoA reductase assay was performed with digitonin-permeabilized cells by the method of Geelen et al. [29], except that the assay was performed on plated cells instead of cells in suspension. The cells in culture dishes of 3.5 cm diameter were incubated with or without different concentrations of AICAR for 30 or 60 min at 37°C and the culture medium was removed and replaced with 200 μl of assay mixture. The assay mixture contained the following components: 16.7 mM imidazole (pH 7.2), 30 mM EDTA, 0.3 mM EGTA, 50 mM KF, 33.5 mM glucose 6-phosphate, 3.4 mM $NADP^+$, 2.2 mM dithiothreitol, 0.33 mM 3-hydroxy-3-methyl-[3-^{14}C]glutaryl-CoA (2 dpm/pmol), 157.5 mU glucose 6-phosphate dehydrogenase and 64 μg of digitonin/mg cell protein. The reaction was stopped by the addition of 20 μl concentrated HCl at 8 min. Blanks were determined in all experiments by stopping the reaction at 0 min. The mevalonate formed during the reaction was left to lactonize for at least 30 min at 37°C. As an internal standard [^{3}H]mevalonolactone was added to all samples. Cells were scraped, centrifuged and aliquots of supernatant were chromatographed on silica gel thin layer plates (Whatman K5D) in acetone/toluene (1:1, v/v), visualized with iodine vapor, scraped into scintillation vials and counted for radioactivity.

2.6. Glucose 6-phosphate assay

Glucose 6-phosphate concentration was determined enzymatically according to Slein [30] as described [10].

2.7. Protein assay

Protein concentration was determined according to Bradford [31] as described [32].

2.8. Statistics

Results are given as means ± S.D. Statistical significance of differences between groups was determined by Student's *t*-test for unpaired data using the StatView software. The minimal level of significance chosen was $P < 0.05$.

3. Results and discussion

3.1. AICAR inhibits glucose-dependent accumulation of L-PK, Spot 14 and FAS mRNAs in hepatocytes in primary culture but does not modify expression of genes which are not stimulated by glucose

The group of Van den Berghe et al. [33], has reported that

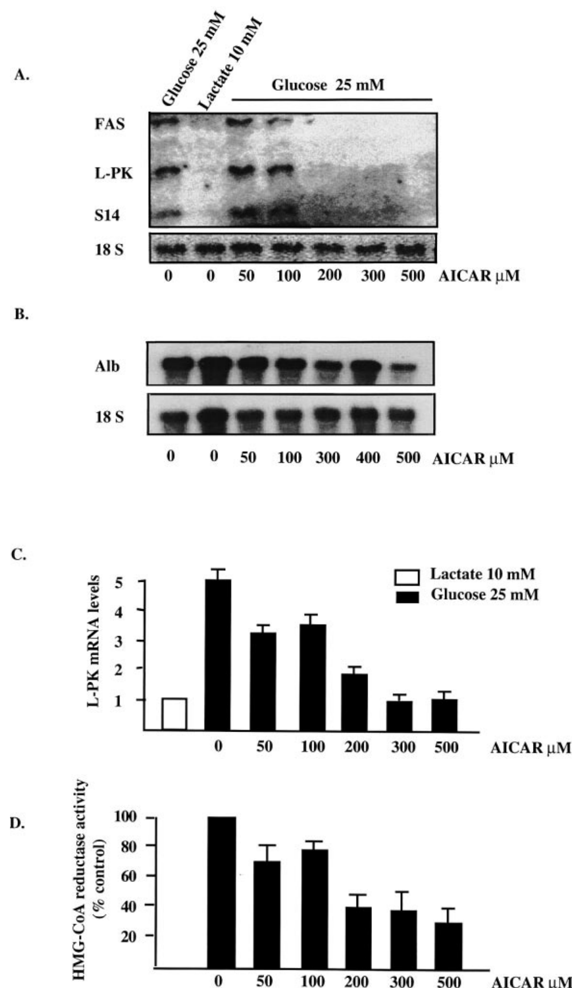

Fig. 1. Effects of AICAR on the level of L-PK, S14, FAS and albumin mRNAs and on HMG-CoA reductase activity in cultured hepatocytes. Mice hepatocytes (A) or rat hepatocytes (B) were cultured either in 10 mM lactate or in 25 mM glucose. Northern blot was performed with total RNA and hybridized with FAS, L-PK, S14 (A) and albumin (B) cDNA probes. C: PhosphorImager quantification of three independent Northern blot analyses of total RNAs from rat hepatocytes hybridized with L-PK cDNA probe. The L-PK RNA levels are expressed as mean ± S.D., and given in arbitrary units. D: HMG-CoA reductase activity in rat hepatocytes in suspension incubated with glucose and increasing AICAR concentrations, expressed as means ± S.D. in percentage of the activity in the absence of AICAR.

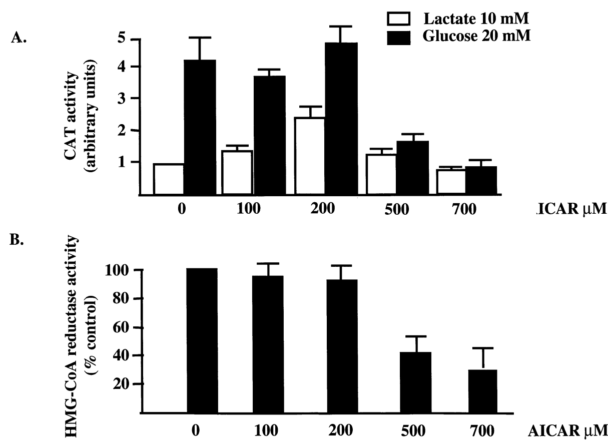

Fig. 2. Effect of AICAR on the glucose-dependent activation of the −183 L-PK/CAT construct and on the HMG-CoA reductase activity in mhAT3F hepatoma cells. A: The mhAT3F cells were transfected with the −183 L-PK/CAT plasmid (containing the GlRE) and incubated for 24 h in 20 mM glucose or 10 mM lactate with various concentrations of AICAR. All values represent the means ± S.D. of at least seven independent experiments. * Significantly higher than in lactate ($P < 0.05$). B: Effect of AICAR on HMG-CoA reductase activity in permeabilized mhAT3F cells. Results were expressed relative to the activity of a control incubation without AICAR in the presence of 20 mM glucose plus insulin (20 nM). All values represent the means ± S.D. of at least seven independent experiments. * Significantly lower than control ($P < 0.05$).

incubation of isolated rat hepatocytes with AICAR provokes inactivation of both acetyl-CoA carboxylase and HMG-CoA reductase activities, and consequently inhibition of fatty acid and cholesterol synthesis. These effects result from the ability of ZMP, the monophosphorylated metabolite of AICAR which accumulates inside the hepatocytes, to stimulate liver AMPK [34]. This stimulation can be explained by the structural analogy of ZMP with AMP [35]. A stimulatory effect of ZMP formed from AICAR on AMPK has also been reported in adipocytes [21,36].

We compared the effect of AICAR in hepatocytes in primary culture on the expression of genes stimulated (L-PK, S14 and FAS) or not (albumin, PEPCK and β-actin) by glucose. Fig. 1 shows that the glucose-dependent accumulation of FAS, L-PK and S14 mRNAs was inhibited by AICAR while the abundance of the glucose-independent albumin mRNA was not. The same was true for PEPCK and β-actin mRNAs whose levels were unaffected by the presence of AICAR in the medium; stimulation of the PEPCK gene by cAMP was normal in the presence of 300 μM AICAR (data not shown). AICAR-dependent inhibition of glucose responsive genes began at 50 μM and was complete at 300 μM. In hepatocytes in primary culture these AICAR concentrations also resulted in the inhibition of HMG-CoA reductase activity, a well known target of AMPK [37], indicating that AMPK is indeed activated in these cells. This result shows that the inhibitory effect of AICAR is specific to glucose-responsive genes and is not due to a non-specific interference with energy production and transcriptional machinery.

3.2. *AICAR inhibits glucose-dependent activation of the L-PK gene promoter in transiently transfected hepatoma cells*

Fig. 2 shows that 500 and 700 μM of AICAR in the culture medium of transfected mhAT3F cells inhibit induction of the −183 L-PK/CAT construct by 20 mM glucose, in parallel with an inhibition of the HMG-CoA reductase activity.

3.3. *The AICAR inhibitory effect requires the presence of a functional glucose response element (GlRE)*

Fig. 3A shows that the inhibitory effect of AICAR requires the presence of the GlRE. This inhibitory effect is not observed with the −150 L-PK/CAT construct lacking the box L4 that is to say the L-PK GlRE [1]. Instead, AICAR slightly stimulated the −150 L-PK promoter activity, as this is also observed in the absence of glucose at 200 μM of AICAR with the −183 L-PK/CAT construct (Fig. 2). Since at this concentration of AICAR the HMG-CoA reductase activity is not reduced in mhAT3F cells, this unexpected stimulatory effect does not seem to involve activation of AMPK, and its mechanism is unknown. The complex assembled on the GlRE includes upstream stimulating factors (USF) 1 and 2 heterodimers [1,23], which are essential components of this complex [32,38,39]. To determine whether AMPK could act directly on USF factors, we tested the effect of AICAR on the activity of the $(MLP)_4$ −54 L-PK/CAT plasmid in which 4 strong USF binding sites are oligomerized upstream of the TATA box containing the proximal fragment of the L-PK promoter [23]. Fig. 3B shows in fact that this construct was stimulated rather than inhibited by AICAR, suggesting that the AMPK acts through a functional GlRE, but not through USF factors which are not glucose response elements [1].

3.4. *The transcriptional inhibition of glucose responsive genes by AICAR is not due to decreased glucose phosphorylation into glucose 6-phosphate*

The first step in the glucose signal pathway through transcriptional machinery is the formation of G 6-P by glucokinase or hexokinase [9,11]. G 6-P concentration in hepatocytes was not affected by AICAR (glucose 25 mM: 1.23 ± 0.65 and glucose 25 mM+AICAR 300–500 μM: 0.93 ± 0.65 nmol/10^6 hepatocytes, P = NS). Consequently, blockade by AICAR of

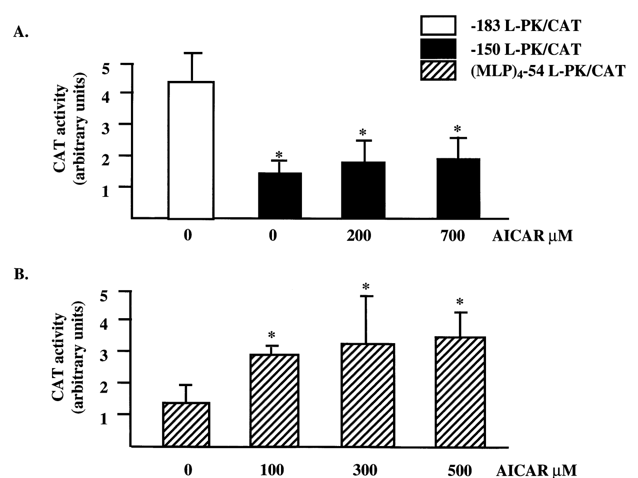

Fig. 3. Effects of AICAR on the activation of the −150 L-PK/CAT and the $(MLP)_4$ −54 L-PK/CAT constructs transfected in mhAT3F hepatoma cells. Cells were cultured under the same conditions as in Fig. 2. A: Transfection with the −150 L-PK/CAT plasmid lacking the GlRE. * Significantly lower than with the −183 L-PK/CAT constructs ($P < 0.05$). B: Transfection with the $(MLP)_4$ −54 L-PK/CAT plasmid containing 4 strong binding sites for USF. * Significantly higher than AICAR 0 μM.

the response of glucose-activated genes to glucose was not a secondary phenomenon due to decreased G 6-P accumulation in cells cultured in high glucose medium. In addition, the effect of AICAR was detected in hepatocytes which synthesize glucokinase, as well as in mhAT3F cells, which instead phosphorylate glucose through other hexokinase isoenzymes [9,40].

3.5. Does AMPK play a transcriptional role in mammals as Snf1p does in yeasts?

The AMPK is considered as a fuel gauge in mammal cells, being activated by a low ATP/AMP ratio which occurs in stressed conditions. The activation of AMPK leads to inactivation by phosphorylation of acetyl-CoA carboxylase, blocks fatty acid synthesis, an energy consuming process, and, by decreasing intracellular malonyl-CoA, permits activation of carnitine palmitoyl-CoA transferase I and fatty acid oxidation [41], thus restoring ATP stores. We suggest in this paper that AMPK could also act at the transcriptional level to permit the switch from a high carbohydrate diet to a low carbohydrate diet or starvation by reducing the expression of glucose-dependent genes in the liver. Glucose starvation in yeast leads to activation of SnfIp and derepression of glucose repressed genes required for metabolism of alternative carbon sources. It is noteworthy to recall that we have previously suggested that glucose could act on the transcriptional machinery through pentose phosphates [11]. Xylulose 5-phosphate has been reported to stimulate a protein phosphatase 2A-like enzyme [42,43]. We also found that okadaic acid, which specifically inhibits protein phosphatases 1 and 2A, blocks the glucose activation of the L-PK promoter in mhAT3F (B. Doiron, unpublished data). Interestingly protein phosphatase 2A has been shown to induce dephosphorylation of AMPK as well as of Snf1p [44]. This is consistent with a dephosphorylation/phosphorylation mechanism involved in the glucose signal pathway through gene transcription.

Finally we think that the inhibitory effect of AICAR does not involve the cAMP inhibitory pathway because AICAR is effective in the mhAT3F cell line which has lost the cAMP response for L-PK gene transcription inhibition [9].

In conclusion, the results presented here suggest that AMPK might act at the transcriptional level to regulate specifically glucose-responsive genes in mammals as Snf1p is involved in the regulation of glucose-dependent genes in yeast. More experiments will be needed to identify the putative partners and targets of AMPK in the glucose signal pathway, and to determine how it could manage to regulate glucose-responsive genes, i.e. to tune the activity of the glucose response complexes assembled on GlREs.

Acknowledgements: We thank Dr. Math J.H. Geelen for his helpful advice about the assay of HMG-CoA reductase activity in digitonin-permeabilized hepatocytes. We thank Mireille Vasseur-Cognet and Sophie Vaulont for the revision of the text.

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