AMP-activated Protein Kinase Inhibits the Glucose-activated Expression of Fatty Acid Synthase Gene in Rat Hepatocytes*

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Although it is now clearly established that a number of genes involved in glucose and lipid metabolism are up-regulated by high glucose concentrations in both liver and adipose tissue, the signaling pathway arising from glucose to the transcriptional machinery is still poorly understood. We have analyzed the regulation of fatty acid synthase gene expression by glucose in cultured rat hepatocytes. Glucose (25 mM) induces an activation of the transcription of the fatty acid synthase gene, and this effect is markedly reduced by incubation of the cells with okadaic acid, an inhibitor of protein phosphatases 1 and 2A. A similar reduction in glucose-activated fatty acid synthase gene expression is obtained by incubation with 5-amino-imidazolecarboxamide riboside, a cell-permeable activator of the AMP-activated protein kinase. Taken together, these results indicate that the glucose-induced expression of the fatty acid synthase gene involves a phosphorylation/dephosphorylation mechanism and suggest that the AMP-activated protein kinase plays an important role in this process. This is the first evidence that implicates the AMP-activated protein kinase in the regulation of gene expression. AMP-activated protein kinase is the mammalian analog of SNF1, a kinase involved in yeast in the transcriptional regulation of genes by glucose.

Although it is now clearly established that a number of genes involved in glucose and lipid metabolism are up-regulated by high glucose concentrations in both liver and adipose tissue (for review, see Refs. 1 and 2), the signaling pathway arising from glucose to the transcriptional machinery is still poorly understood. There is general agreement on the fact that glucose has to be metabolized to stimulate the transcription of lipogenic-related genes such as L-pyruvate kinase, fatty acid synthase (FAS), acetyl-CoA carboxylase (ACC), and S14 (3–5). In previous papers, we have proposed that glucose-6-phosphate could be the signal metabolite for the glucose-induced FAS transcription (3, 5, 6) and therefore for other genes belonging to the same class. This hypothesis was challenged by Doiron et al. (7) who proposed that the signal metabolite was xylulose-5-phosphate. However, whatever the metabolite, the link between the glucose signal and the activation of gene transcription remains unknown.

Phosphorylation/dephosphorylation processes are one of the major mechanisms involved in the regulation of glucose and lipid metabolism both at the cellular and molecular levels in eukaryotic cells. It is now well established that many transcription factors have their activity regulated by phosphorylation through a modification of their DNA binding activity, their transactivating capacity, or their subcellular localization (8).

In cultured hepatocytes, it has been shown that okadaic acid, an inhibitor of phosphatases 1 and 2A, led to the inhibition of glucose stimulation of S14 gene transcription (9). Further experiments with calcycin, a much more potent inhibitor of protein phosphatase 1 than okadaic acid, suggest that protein phosphatase 2A may play the major role in the glucose effect. Finally, since the calcium ionophore A23187, an activator of Ca2+/calmodulin-dependent protein kinase (CaM kinase) inhibited the glucose effect, it was concluded that CaM kinase and protein phosphatase 2A were implicated in the glucose regulation of S14 gene transcription (9).

In the present work, we show that AMP-activated protein kinase (AMPK) is involved in the regulation by glucose of FAS gene expression in cultured hepatocytes. It must be pointed out that AMPK is the mammalian equivalent of SNF1, a protein kinase complex essential for glucose-regulated gene expression in yeast through the modulation of the transcriptional activity of nuclear factors.

EXPERIMENTAL PROCEDURES

Animals—Animal studies were conducted according to the French Guidelines for the Care and Use of Experimental Animals. Female Wistar rats (200–300 g body weight) from Iffa-Credo, (L’Arbresle, France) were used. They were housed in plastic cages at a constant temperature (22 °C) with light from 0700 h to 1900 h for at least one week before the experiments.

Isolation and Primary Culture of Hepatocytes—Hepatocytes were isolated by the collagenase method (10). Cell viability was assessed by the Trypan Blue exclusion test and was always higher than 85%.

Hepatocytes were seeded at a density of 8 × 10^6 cells/dish in 100-mm Petri dishes in medium M199 with Earle’s salts (Life Technologies, Inc., Paisley, UK) supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin, 0.1% (w/v) bovine serum albumin, 2% (v/v) Ultroser G (IBF, Villeneuve la Garenne, France), 100 nM dexamethasone (Sigma), 1 nM insulin (Actrapid, Novo-Nordisk, Copenhagen, Denmark), 100 nM triiodothyronine (T3) (Sigma). After cell attachment (4 h), the hepatocytes were cultured for 16–18 h in the presence of 5 mM glucose in a medium similar to the seeding medium but free of Ultroser and albumin and containing 100 nM insulin. The presence of dexamethasone, T3, and insulin in the culture medium ensures a maximal activity of glucokinas, necessary for a full glucose effect on FAS gene expression (11, 12).

REFERENCE

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§ The abbreviations used are: FAS, fatty acid synthase; ACC, acetyl-CoA carboxylase; AICAR, 5-aminooimidazole-4-carboxamide-1-β-D-ribofuransyl; AMPK, AMP-activated protein kinase; CaMK, Ca2+/calmodulin-dependent kinase; I-PK, L-pyruvate kinase; PEPCCK, phosphoenolpyruvate carboxykinase; PKA, protein kinase cAMP dependent; SAMS, the synthetic peptide substrate with the amino acid sequence HMRASMSGHLVLKRR; BtγcAMP, dibutyryl cyclic AMP; ZMP, 5-amino-4-imidazolecarboxamide ribotide.
After 16–18 h, the culture medium was removed and hepatocytes were then cultured for 1, 3, or 6 h in the presence of either 5 or 25 mM glucose, hormones, and various compounds including 10–100 nM okadaic acid (Sigma), 50 μM 87 (protein kinase A and C inhibitor) (Sigma), 1 μM A23187 (activator of CaM kinases) (Sigma), 1 μM KN62 (inhibitor of CaM kinase II) (Sigma), 50–500 μM 5-aminoimidazole-4-carboxamide-1-β-ribofuranosyl (AICAR) (Sigma) (activator of AMPK).

Metabolite Concentration Assay—Cells were scrapped into 0.5 ml of 6% (v/v) ice-cold HClO₄ in less than 5 s after removing the culture medium. The concentrations of metabolites including glucose-6-phosphate, lactate, AMP, ADP, and ATP were assayed enzymatically as described previously (13).

Measurement of AMPK Activity—Hepatocytes were directly lyzed in the culture medium by adding 1.5 ml of buffer A (final concentrations of 50 mM Tris-HCl, pH 7.5, 50 mM NaF, 5 mM sodium pyrophosphate, 1 mM EDTA, 10% glycerol, 1 mM dithiothreitol) + 1% Triton X-100. The cellular debris were pelleted by centrifugation at 4000 × g for 15 min, and the resulting supernatant was removed, adjusted to 10% with PEG 6000 (Appligene, Illkirch, France), and kept on ice for 20 min. Following further centrifugation (7000 × g, 15 min), the pellet of proteins was resuspended in 400 μl of buffer A. Aliquots (5 μl) were used to assay the AMPK activity by the SAMS peptide phosphorylation assay in the presence of saturating concentrations of 5′-AMP (200 μM) as described previously (14).

Penetration of AMPK—AMPK was partially purified from rat liver up to and including the DEAE-Sepharose ion-exchange step as described previously (15). AMPK was further purified by immunoprecipitation using antibodies raised against the AMPKα1 subunit bound to protein A-Sepharose. The immune complex was washed thoroughly with buffer A, and the resin was suspended in buffer A as a 50% (v/v) slurry and used for phosphorylation reactions.

Isolation of Total RNA and Northern Blot Hybridization—Total cellular RNAs were extracted from hepatocytes using the guanidine thiocyanate method (16) and prepared for Northern blot hybridization as described previously (17). Labeling of each probe was performed by random priming (Rediprime labeling kit, Amersham Pharmacia Biotech). Autoradiograms of Northern blots were scanned and quantified using an image processor program.

FAS and PEPCK cDNAs were as described previously (17). Albumin, β-actin, and glucokinase cDNAs were gifts of kindness, respectively, from Drs. J. L. Danon (Meudon, France), B. Spiegelman (Boston, MA), and P. Ineydijian (Geneva, Switzerland). Each Northern blot was hybridized with a ribosomal 18 S cDNA to verify that equivalent amounts of total RNA were loaded in each lane.

Nuclear Run-on Transcription Assay—Nuclear isolation and nuclear run-on transcription experiments were performed as described by Daigui et al. (18).

Statistical Analysis—Results are expressed as means ± S.E. Statistical analysis was performed with Student’s t test for unpaired data. When quantified, FAS mRNA concentrations were normalized with respect to the 18 S hybridization signal.

RESULTS AND DISCUSSION

Okadaic Acid Inhibits the Glucose Effect on FAS Gene Expression—To investigate whether a phosphorylation/dephosphorylation mechanism is involved in the glucose signaling pathway, we have used okadaic acid, a potent inhibitor of type 1 and 2A protein phosphatases (19), the most abundant phosphatases present in the liver (20). Okadaic acid has been shown to increase the phosphorylation state of a number of proteins including ACC or 1-PK in rat hepatocytes, leading to increased glucose output and reduced glycolysis and lipogenesis (20). A major problem faced by studies using okadaic acid in whole cells is that, due to the widespread role of protein phosphorylation, long-term incubations could lead to secondary effects not related to glucose signaling. To minimize this problem in the present study, okadaic acid was added at the same time as 25 mM glucose in cultured hepatocytes, and FAS mRNA concentration measurements were performed after only 3 h. Okadaic acid at a dose of 10 nM decreases glucose-induced FAS expression by 50%, with a maximal effect obtained at 50 nM (Fig. 1). Moreover, the inhibitory effect of okadaic acid is not due to a general impairment of transcription mechanisms since (i) the expression of a control gene, β-actin, is not affected in the same experiments (Fig. 1), and (ii) the inhibitory effect of okadaic acid on PEPCK mRNA abundance in hepatocytes, a phenomenon previously described (21), can still be counteracted by glucagon (Fig. 1).

Previous studies have shown that glucose metabolism is a prerequisite for glucose action on FAS gene expression (3, 5). It was important, therefore, to ensure that in these experiments, okadaic acid did not directly affect glucose metabolism. In fact, glucose-6-phosphate concentrations measured 1 h after 10 nM okadaic acid addition, a dose which markedly inhibits FAS gene expression, were rather slightly increased (control, 2.20 ± 0.14 nmol/10⁶ hepatocytes (n = 3); okadaic acid-treated, 2.76 ± 0.13 nmol/10⁶ hepatocytes (n = 3)). Furthermore, lactate produced from glucose in the culture medium 3 h after 10 nM okadaic acid addition was unchanged (control, 1.50 ± 0.02 μmol/10⁶ hepatocytes (n = 3); okadaic acid-treated, 1.48 ± 0.1 μmol/10⁶ hepatocytes (n = 3)). These data rule out an indirect effect of okadaic acid through an impairment of glucose metabolism.

Our results are reminiscent of experiments showing that S14 gene transcription in cultured hepatocytes is inhibited by incubation with 5 nM okadaic acid with a maximal effect at 50 nM (9). Due to the fact that okadaic acid is not selective between phosphatases 1 and 2A, we cannot conclude whether phosphatase 2A alone is involved as shown for glucose induction of S14 gene transcription (9). Nevertheless, these experiments demonstrate that the stimulatory effect of glucose on FAS gene expression does involve a dephosphorylation mechanism.

An Activator of AMPK Opposes the Glucose Effect on FAS Gene Expression—Having established that protein phosphatases are involved in the glucose signaling pathway, we next investigated which protein kinases were involved. Cultured hepatocytes were incubated in the presence of a number of different compounds to modulate the activity of various protein kinases (see “Experimental Procedures”). In all cases, except
and 500 different inhibitors and activators of kinases: 1 mM hormones (100 nM insulin, 100 nM dexamethasone, 100 nM T3). Cells were cultured for 16–18 h in the presence of 5 mM glucose and hormones in the absence or in the presence of increasing concentrations of AICAR (50–500 μM) and then sampled either for mRNA extraction or for AMPK activity measurement. Top panel, total RNA were extracted and analyzed for expression of FAS and glucokinase genes. A representative Northern blot from two experiments is shown. Bottom panel, AMPK activity expressed in cpm incorporated into SAMS peptide/min/μg of protein. The results are presented as the mean ± S.E. for two experiments with triplicates in each experiment.

Fig. 2. Effect of kinase activators and inhibitors on glucose-induced FAS gene expression in cultured hepatocytes. Hepatocytes were cultured for 16–18 h in the presence of 5 mM glucose and hormones (100 nM insulin, 100 nM dexamethasone, 100 nM T$_3$). Top panel, cells were then treated for 6 h with 15 or 25 mM glucose and hormones in the absence or in the presence of different inhibitors and activators of kinases: 50 μM H$_7$, an inhibitor of both kinases A and C; 1 μM A23187, an activator of CaM kinases; and 1 μM KN62, an inhibitor of CaM kinase II. Middle panel, cells were then treated for 6 h with 15 or 25 mM glucose and hormones in the absence or in the presence of different inhibitors and activators of kinases: 1 μM A23187; 50 μM H$_7$; and 500 μM AICAR, an activator of AMP-activated protein kinase. Bottom panel, cells were then treated for 6 h with 25 mM glucose and hormones in the absence or in the presence of 100 μM Bt$_2$cAMP. Total RNA were extracted and analyzed for the concentration of FAS, glucokinase, and PEPCK mRNAs. Loading of equivalent amounts of total RNA was checked by hybridization with a ribosomal 18 S cDNA probe. These Northern blots are representative of three different culture experiments.

when Bt$_2$cAMP was used, hepatocytes were incubated with a sub-maximal concentration of glucose (15 mM) to be able to detect both an increase or decrease in FAS gene expression by the various drugs.

We have analyzed the effects of H$_7$, an inhibitor of both cAMP-dependent protein kinase (PKA) and protein kinase C (22), A23187, a calcium ionophore that activates CaM kinases (23) and KN62, a specific inhibitor of CaM kinase II (24). In the presence of these various compounds (Fig. 2, top and middle panels), there was no significant change in the glucose-induced FAS gene expression. In contrast, AICAR, a compound which has been shown to activate AMPK in a number of cell types, including primary rat adipocytes and hepatocytes (14, 25) and Bt$_2$cAMP (an activator of PKA), strongly reduces the stimulating effect of glucose on FAS gene expression (Fig. 2, middle and bottom panels). The inhibitory effect of AICAR is not a general effect on gene transcription as shown by the fact that glucokinase gene expression is not modified by the compound (Fig. 2, middle panel). Similarly, the inhibitory effect of cAMP on FAS gene expression was specific since PEPCK gene expression was strongly stimulated by Bt$_2$cAMP (Fig. 2, bottom panel). These results suggest a potential involvement of AMPK and PKA in the glucose signaling pathway.

We have already shown that activation of PKA in adipose tissue counteracted the glucose effect on FAS gene expression (26). Similarly, activation of PKA inhibited glucose-induced expression of the S14 (27) and L-PK (28) genes, although for the former, it has been attributed to an effect on glucose metabolism (9) and for the latter to an effect on glucokinase activity. Indeed, the cAMP inhibitory effect on L-PK gene expression was lost in a hepatoma cell line expressing hexokinase II rather than glucokinase (29). In the present study, Bt$_2$cAMP strongly increases glucose-6-phosphate concentration in hepatocytes (result not shown), ruling out an indirect metabolic effect of PKA activation. If PKA was the kinase partner of the glucose signaling system, it would be anticipated that its inactivation should stimulate the FAS gene expression in the presence of 15 mM glucose. Since an inhibitor of PKA (H$_7$) has no stimulatory effect on FAS gene expression (Fig. 2), this may indicate that PKA inhibits FAS gene expression by a mechanism that is unrelated to the glucose activating system. Moreover, in hepatocytes cultured in the presence of 5 mM glucose which express FAS at a low level, cAMP concentration should be already low due to the presence of insulin. This is backed up by the observation that PEPCK gene expression, which is dependent upon the activation of PKA, is barely detectable in these culture conditions (results not shown). This strongly suggests that PKA is not involved in the inhibition of FAS gene expression at low glucose concentrations. This hypothesis is consistent with the finding that a cAMP DNA response element has been localized at –99 to –92 in the FAS promoter (30), a region which bears no relationship with known glucose-response elements (for review, see Ref. 1).

Following uptake into the cell, AICAR is metabolized into ZMP, a relatively potent activator of AMPK (14, 25, 31). Progressive inhibition of glucose-induced FAS gene expression by increasing concentrations of AICAR was indeed paralleled by an increased activity of AMPK (Fig. 3). In the same culture experiments, glucokinase gene expression that is not controlled by glucose but by insulin (32) was not affected (Fig. 3). Activa-
Hepatocytes were cultured for 16–18 h in the presence of 5 mM glucose and a submaximal dose of AICAR in cultured hepatocytes. Cells were then treated for 6 h with increasing concentrations of glucose (15–35 mM) in the absence or in the presence of 200 μM AICAR. Cells were then treated for 1 h with 5 or 25 mM glucose and hormones in the absence or in the presence of 250 μM AICAR. Nuclei were isolated from cultured cells as described under “Experimental Procedures.” The radiolabeled transcripts were hybridized to dot-blotted cDNAs for FAS, glucokinase, and to pBluescript II KS⁺, the vector into which the inserts were cloned. The blot is representative of two experiments.

**Table I**

- **Glucose**
  - 5 mM Glucose
  - 25 mM Glucose
  - 25 mM Glucose + 500 AICAR μM
  - **AMPK activity (nmol/10^6 hepatocytes)**
    - 3.13 ± 0.08
    - 3.22 ± 0.09
  - **ADP (nmol/10^6 hepatocytes)**
    - 1.40 ± 0.25
  - **AMP (nmol/10^6 hepatocytes)**
    - 1.18 ± 0.22

**Fig. 4.** Effect of glucose on FAS gene expression in the presence of a submaximal dose of AICAR in cultured hepatocytes. Hepatocytes were cultured for 16–18 h in the presence of 5 mM glucose and hormones (100 nM insulin, 100 nM dexamethasone, 100 nM T₃). Cells were then treated for 6 h with increasing concentrations of glucose (15–35 mM) in the absence or in the presence of 200 μM AICAR. The results are presented as a representative Northern blot (top panel) or in arbitrary densitometric units as the mean of two experiments with duplicates in each experiments (bottom panel).

**Fig. 5.** Effect of glucose and AICAR on FAS and glucokinase gene transcription in cultured hepatocytes. Hepatocytes were cultured for 16–18 h in the presence of 5 mM glucose and hormones (100 nM insulin, 100 nM dexamethasone, 100 nM T₃). Cells were then treated for 1 h with 5 or 25 mM glucose and hormones in the absence or in the presence of 500 μM AICAR. AMPK activity is expressed as cpm incorporated into SAMS peptide/min/μg of protein. Results are presented as mean ± S.E. for three experiments. * Differences significant for p < 0.001 when compared with hepatocytes cultured in the presence of 25 mM glucose alone.

**Activation of AMPK Decreases Glucose-induced FAS Gene Expression by a Transcriptional Mechanism.** To assess whether the decreased expression of FAS gene by the activation of AMPK was due to a transcriptional and/or a post-transcriptional mechanism, run-on experiments were performed. Nuclei were isolated from hepatocytes cultured for 1 h in the presence of 5 or 25 mM glucose and in the absence or presence of AICAR (250 μM). Glucose at the high concentration clearly increases FAS gene transcription (Fig. 5). This increase was strongly (but not totally) repressed in the presence of AICAR, whereas glucokinase transcription was neither affected by glucose nor by AICAR (Fig. 5). It is therefore clear that the decreased FAS gene expression when AMPK is activated involves a transcriptional mechanism. Nevertheless, since the inhibitory effect of AICAR on FAS gene transcription is not total whereas the effect on FAS mRNA concentration is drastic, this might indicate an effect of AMPK activation on FAS mRNA half-life. On the other hand, the partial inhibitory effect of AICAR on FAS gene transcription might suggest that other kinases are also involved in the glucose-signaling system.

The bulk of these studies strongly suggests that AMPK interferes with the glucose-signaling system for the FAS gene transcription. In addition to our findings, there are a number of features that provide clues for a role of AMPK in gene regulation in response to glucose. First, AMPK phosphorylates and inactivates ACC and 3-hydroxy-3-methylglutaryl-CoA reductase, resulting in the inhibition of both lipogenesis and cholesterol synthesis (33). These two pathways utilize acetyl-CoA as a substrate that, for a large part, originates from glucose. The action of AMPK on FAS gene expression would thus allow the potential for the coordinate regulation of lipid synthesis from glucose in both the short and long term. Second, and perhaps most interesting, is the finding that AMPK is structurally and functionally related to the yeast SNF1 protein kinase complex (34). AMPK consists of three subunits, a catalytic subunit, α (63 kDa), and two noncatalytic subunits, β (30 kDa) and γ (36 kDa). AMPK α subunit is 46% identical to yeast Snf1p, and this similarity increases to 60% within the kinase catalytic domains (35, 36). The γ subunit is 35% identical to yeast Snf4p (37), which is required for the protein kinase activity of Snf1p, just as the mammalian γ subunit is essential for the maximal kinase activity of the catalytic α subunit (38). The AMPK β subunit is related to the yeast Sip family which interacts with Snf1p (37). Studies using synthetic peptides as substrates have revealed that both AMPK and SNF1 share very similar recognition motifs for phosphorylation, and moreover, they both phosphorylate and inactivate acetyl-CoA carboxylase in vivo (35). Most importantly, the activity of SNF1 is essential for the transcriptional activation of glucose-repressed genes in Saccharomyces cerevisiae (39). This is achieved through the phosphorylation of a transcription factor, depending upon the medium glucose concentration (40). Finally, it can be underlined that, as described for the glucose control of gene expression in mammals, the phosphorylation of glucose by a hexokinase is an essential step for the transcriptional effect of glucose in yeast (41). It is then tempting to speculate that the general mechanisms involved in the regulation of gene expression by glucose in mammals bear similarity with those described in yeast.
Glucose and Its Signal Metabolite Do Not Modulate the Concentration of AMP or the Activity of AMPK—It has been suggested that SNF1 activity varies in response to glucose in yeast (35). One obvious possibility was that a high glucose concentration decreases the activity of AMPK. AMP activates AMPK through several independent mechanisms: allosteric activation of AMPK, stimulation of an AMPK kinase (AMPK is activated by phosphorylation), and inhibition of the deactivation of AMPK by PP-2C (42). A decreased AMPK activity in the presence of a high glucose concentration could be achieved (i) by decreasing the concentration of AMP, (ii) by altering the phosphorylation state of the enzyme, or (iii) by a direct allosteric interaction between the glucose signal metabolite and AMPK.

To test the first hypothesis, we have measured the concentration of AMP in the presence of 5 or 25 mM glucose. As can be seen from Table I, a high glucose concentration does not modify AMP, ADP, or ATP concentrations.

We have then assessed whether AMPK activity, and hence its phosphorylation state, was modified in the presence of a stimulating glucose concentration in cultured hepatocytes. This measurement relies on the fact that a change in the phosphorylation state of AMPK in the hepatocyte would be preserved in our assay conditions because of the presence of phosphatase inhibitors. As summarized in Table I, a high glucose concentration had no inhibitory effect on AMPK activity, whereas AICAR clearly increases its activity.

A last possibility was that the glucose signal metabolite could modify AMPK activity. An antagonism between glucose-6-P and AMP, ADP, or ATP concentrations.

The main stimulator of FAS gene expression in adipose tissue and in the liver is glucose with the clear identification of the transcription factors involved in the glucose response.

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