5-Aminoimidazole-4-Carboxamide Riboside Mimics the Effects of Insulin on the Expression of the 2 Key Gluconeogenic Genes PEPCK and Glucose-6-Phosphatase

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Insulin regulates the rate of expression of many hepatic genes, including PEPCK, glucose-6-phosphatase (G6Pase), and glucose-6-phosphate dehydrogenase (G6PDHase). The expression of these genes is also abnormally regulated in type 2 diabetes. We demonstrate here that treatment of hepatoma cells with 5-aminoimidazole-4-carboxamide riboside (AICAR), an agent that activates AMP-activated protein kinase (AMPK), mimics the ability of insulin to repress PEPCK gene transcription. It also partially represses G6Pase gene transcription and yet has no effect on the expression of G6PDHase or the constitutively expressed genes cyclophilin or β-actin. Several lines of evidence suggest that the insulin-mimetic effects of AICAR are mediated by activation of AMPK. Also, insulin does not activate AMPK in H4IIE cells, suggesting that this protein kinase does not link the insulin receptor to the PEPCK and G6Pase gene promoters. Instead, AMPK and insulin may lie on distinct pathways that converge at a point upstream of these 2 gene promoters. Investigation of the pathway by which AMPK acts may therefore give insight into the mechanism of action of insulin. Our results also suggest that activation of AMPK would inhibit hepatic gluconeogenesis in an insulin-independent manner and thus help to reverse the hyperglycemia associated with type 2 diabetes.
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One of the most important actions of insulin is the regulation of gene expression. Insulin has been shown to regulate the expression of >100 genes (1). Two of the best-studied insulin-responsive genes are PEPCK (2–6) and glucose-6-phosphatase (G6Pase) (7–9). The products of these genes are key enzymes of hepatic gluconeogenesis, and their abnormal regulation has been associated with the development of type 2 diabetes and other metabolic disorders (10–15). The transcription of both genes is induced by glucagon (via cAMP) and glucocorticoids and is inhibited by insulin (7,14,16–18). The molecular mechanisms underlying the repression of PEPCK gene transcription by insulin have been studied in great detail. Indeed, the activities of almost all of the signaling molecules known to be regulated by insulin have been manipulated in some fashion to determine their role in this action of insulin. However, to date, only the molecules involved in the generation of the putative insulin second messenger, phosphatidylinositol 3,4,5-trisphosphate (PIP3), have been conclusively demonstrated to be required for repression of PEPCK transcription by insulin (19–21). This process is not affected by the presence of inhibitors of the Ras–mitogen-activated protein (MAP) kinase, the p70 S6 protein kinase, or p38 MAP kinase pathways; dominant-negative forms of protein kinase B (PKB), atypical protein kinase C (PKC), or the small G protein Rac; or the downregulation of conventional PKC (19–23). In addition, not all of the DNA sequences in the PEPCK promoter required for full repression of this gene by insulin have been characterized, and no insulin-regulated transcription factor has been identified that is required for the inhibition of this gene promoter by insulin. Similarly, the proteins that bind to and regulate the G6Pase gene promoter are not fully understood. However, the signaling pathway from the insulin receptor to the G6Pase gene promoter also requires the generation of PIP3 but is not affected by inhibitors of the Ras-MAP kinase pathway (24) or overexpression of dominant-negative PKB (P.A.L., C.S., unpublished data).

Previously, we reported that the transcription of hepatic PEPCK is repressed by hydrogen peroxide or sodium arsenite (23). In addition, exposure of hepatoma cells to glucose (25), okadaic acid, or phorbol ester (22) or treatment of hepatocytes with troglitazone (6) mimic this action of insulin. Repression by phorbol ester is mediated by conventional PKC activation, but the mechanisms by which gene repression is

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8CPT-cAMP, 8-(4-chlorophenylthio)-cAMP; AICAR, 5-aminoimidazole-4-carboxamide riboside; AMPK, AMP-activated protein kinase; AMPKK, AMP-activated protein kinase kinase; CAT, chloramphenicol acetyltransferase; DMEM, Dulbecco's modified Eagle's medium; DTT, dithiothreitol; F16BPase, fructose-1,6-biphosphatase; G6Pase, glucose-6-phosphatase; G6PDHase, glucose-6-phosphate dehydrogenase; IRS, insulin response sequence; MAP, mitogen-activated protein; PIP3, phosphatidylinositol 3,4,5-trisphosphate; PKB, protein kinase B; PKC, protein kinase C; PMSF, phenylmethylsulfonyl fluoride; RPA, RNase protection assay; ZMP, AICA ribotide.

achieved by the other agents are unknown. However, the generation of PIP3 is not required by any of these agents, whereas the activation of p38 MAP kinase is not required for the action of hydrogen peroxide or sodium arsenite (23). In addition, all of these agents exert their effects independently of insulin.

AMP-activated protein kinase (AMPK) is the central component of a protein kinase cascade that monitors cellular energy charge (26), being activated by a rise in the cellular AMP:ATP ratio (27). AMPK appears to act as a "metabolic master switch" (28), regulating metabolism both via direct phosphorylation of metabolic enzymes and via effects on gene expression. The ***Saccharomyces cerivisiae*** homolog of AMPK is the SNF1 complex, which regulates gene expression in response to the availability of glucose (26). AMPK is activated by treatments that deplete ATP, such as heat shock or arsenite in hepatocytes (29), exercise in skeletal muscle (30), ischemia in heart (31), and glucose deprivation in pancreatic β-cell lines (32). AMPK can also be artificially activated in intact cells by treatment with the nucleoside 5-aminoimidazole-4-carboxamide riboside (AICAR) (33,34). This nucleoside is taken up and accumulates inside the cell as the monophosphorylated nucleotide, AICA ribotide (ZMP). ZMP mimics all of the effects of AMP on the AMPK system, including stimulation of phosphorylation by the upstream kinase, AMP-activated protein kinase kinase (AMPKK) (33). This method of activating AMPK has the advantage that it does not disturb the levels of ATP, ADP, or AMP (33,34), and therefore any changes seen are not merely due to depletion of ATP.

Recently, it has been pointed out that activation of AMPK would be expected to reverse many of the metabolic abnormalities associated with type 2 diabetes (28). A corollary of this is that a subset of type 2 diabetes might be due to reduced expression or a low activation state of AMPK (28). In view of the fact that both insulin and AMPK are "metabolic switches" acting on other aspects of carbohydrate and lipid metabolism, we examined the effects of AMPK activation on expression of enzymes of gluconeogenesis, one of the key actions of insulin. Here, we report that activation of AMPK partially mimics the effects of insulin on hepatic gluconeogenesis.

RESEARCH DESIGN AND METHODS

Radioisotopes were obtained from Amersham (Chalfont, Bucks, U.K.) ([γ-^{32}P]ATP) and ICN (Thame, Oxfordshire, U.K.) ([α-^{32}P]UTP and [^{3}H]sodium acetate). Insulin was purchased from Novo Nordisk (Crawley, West Sussex, U.K.), 8-(4-chlorophenylthio)-cAMP (8CPT-cAMP) from Boehringer Mannheim/Roche Diagnostics (Lewes, East Sussex, U.K.), and RNase Protection Assay Kit II from AMS Biotech/Ambion (Austin, TX). All other chemicals were of the highest grade available.

Cell culture, hormone treatments, and CAT assay. The isolation of the H4IIE rat hepatoma–derived stable transfectant, HL1C, which contains the PEPCK promoter sequence from –2100 to 69 ligated to the chloramphenicol acetyltransferase (CAT) reporter gene, has been described previously (35). HL1C (and H4IIE) cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 1,000 g/l glucose, 2.5% (vol/vol) fetal calf serum, and 2.5% newborn calf serum, as described previously (35). Hormone and inhibitor treatments were carried out in serum-free DMEM for the times and at the concentrations indicated in the figure legends. Cells were harvested by tryptic digestion and sonicated in 400 µl of 250 mmol/l Tris/HCl (pH 7.8) before CAT activity was determined (35).

Synthesis and labeling of oligonucleotides. Two oligonucleotides, PC28 (5′-GGAGAGAGGCAGGGACTCTGGTGCCACC-3′) and ACT25 (5′-GGGTGTG GACCGGGACGGAGGAGCT-3′), were synthesized and purified by MWG-Biotech (Milton Keynes, U.K.). They were designed to be complementary to positions 102 to 129 and 42 to 67, relative to the transcription start site, in the PEPCK and β-actin genes, respectively. The oligonucleotides were 5′ end-labeled with [γ-^{32}P]ATP (specific activity ~10^9 cpm/µg) before their use in a primer extension assay (35).

RNA isolation, primer extension analysis (PEPCK), and RNase protection assay (G6Pase and G6PDHase). H4IIE cells were serum-starved overnight and treated with hormone/inhibitor for the times and at the concentrations indicated in the figure legends. Total cellular RNA (~100 µg/10^6 cells) was isolated using TriReagent (Sigma, Poole, Dorset, U.K.) following the manufacturer's instructions. A primer extension assay was used to analyze PEPCK and β-actin mRNA accumulation as described previously (35). An RNase protection assay (RPA) was performed to determine the relative proportions of G6Pase, glucose-6-phosphate dehydrogenase (G6PDHase), and cyclophilin mRNA in each sample. The sequences of the rat G6Pase cDNA from nucleotide 117 to 291 and the rat G6PDHase from nucleotide 44 to 273 were amplified from rat liver cDNA by polymerase chain reaction, and the resultant products were cloned into the plasmid pCR2.1-TOPO (Invitrogen, Groningen, the Netherlands). The ***Apa***I to ***Eco***RV fragment of the G6Pase plasmid was removed by restriction digestion, and the plasmid was religated. The plasmids were cut with ***Bst***XI (G6Pase) or ***Spe***I (G6PDHase) to produce linear DNA templates, whereas pTRI-cyclophilin linear plasmid (Ambion) was used as the cyclophilin DNA template. G6Pase, G6PDHase, and cyclophilin RNA probes were synthesized by in vitro transcription in the presence of [α-^{32}P]UTP (0.33 µCi/nmol) using T7 RNA polymerase as directed by MaxiScript Kit (Ambion). Of each probe, 3,000 cpm was hybridized overnight at 42°C with 10 µg total RNA, as directed by the RPA II Kit (Ambion). In brief, the probes and mRNA of interest (in the total RNA sample) are hybridized to form double-stranded RNA complexes, and single-stranded RNA was removed by digestion with RNase. Samples were analyzed on an 8 mol/l urea, 5% polyacrylamide gel; the intensity of the band is proportional to the amount of mRNA. Band intensity was quantitated on a Fuji phosphorimager (Raytek Scientific, Sheffield, U.K.), and data were calculated as a ratio of G6Pase/cyclophilin or G6PDHase/cyclophilin and presented as fold activation, where the intensity of control (no serum) samples was set at 1.

Transient transfection. Plasmids encoding PEPCK promoter-CAT reporter (pPL32, containing nucleotides from –440 to 32 relative to the transcription start site of the PEPCK promoter linked to CAT cDNA [36]), glucocorticoid receptor (RSV-GR, from Keith Yamamoto, San Francisco, CA), or control (RSV-neo) were transiently transfected into H4IIE cells as described previously (37). Cells were incubated for 20 h with hormones as indicated in the figure legends and then harvested by tryptic digestion and sonicated in 200 µl of 250 mmol/l Tris/HCl (pH 7.8) before CAT activity was determined (35).

Preparation of cell extract for kinase assays. H4IIE cells were incubated in serum-free medium with hormones and inhibitors for the times and at the concentrations indicated in the figure legends. Cells were then scraped into ice-cold lysis buffer (50 mmol/l Tris-HCl, pH 7.4 at 4°C, 50 mmol/l NaF, 1 mmol/l sodium pyrophosphate, 1 mmol/l EDTA, 1 mmol/l EGTA, 1 mmol/l dithiothreitol [DTT], 0.1 mmol/l benzamidine, 0.1 mmol/l phenylmethylsulfonyl fluoride [PMSF], 0.2 mmol/l sodium vanadate, 250 mmol/l mannitol, 1% Triton X-100, and 5 µg/ml soybean trypsin inhibitor). Cell debris was removed by centrifugation at 13,000***g*** for 5 min, and the protein concentration was determined, using bovine serum albumin as an internal standard, by the method of Bradford.

Immunoprecipitation and assay of AMPK. Cell extract (0.1 mg) was incubated for 2 h at 4°C on a shaking platform with protein G sepharose conjugated to anti-AMPK1 (α1) and AMPK2 (α2). The immunocomplexes were pelleted and washed 4 times with 1.0ml buffer A (50mmol/l Tris-HCl, pH 7.4 at 4°C, 1mol/l NaCl, 50 mmol/l NaF, 1 mmol/l sodium pyrophosphate, 1 mmol/l EDTA, 1 mmol/l EGTA, 1 mmol/l DTT, 0.1 mmol/l benzamidine, 0.1 mmol/l PMSF, 0.2 mmol/l sodium vanadate, 1% Triton X-100, and 5 µg/ml soybean trypsin inhibitor) and once with 1.0ml HEPES-Brij buffer (50mmol/l HEPES, pH7.4, 1 mmol/l DTT, 0.02% [vol/vol] Brij-35). The immunoprecipitated kinase activity was assayed by a modification of the method of Davies et al. (38), that is, at 30°C, in the presence of 5mmol/l $MgCl_2$, 0.2mmol/l [γ-^{32}P]ATP (2 × 10^6 cpm/nmol), 0.2mmol/l AMP, and 200µmol/l AMARA peptide. One unit of kinase activity is that amount catalyzing the phosphorylation of 1 nmol substrate in 1 min.

RESULTS

PEPCK gene transcription is induced by glucocorticoids and cAMP-elevating agents in intact liver, isolated hepatocytes, and hepatoma cells, whereas insulin blocks all of these effects (Fig. 1) (3,6,18,39). A number of other reagents also reduce PEPCK gene transcription in a dominant fashion, thereby exhibiting an insulin-mimetic effect. We now report that AICAR also mimics this effect of insulin. Repression of endogenous PEPCK gene transcription is achieved in a dose-dependent fashion with initial effects between 150 and 200 µmol/l and almost complete repression obtained at

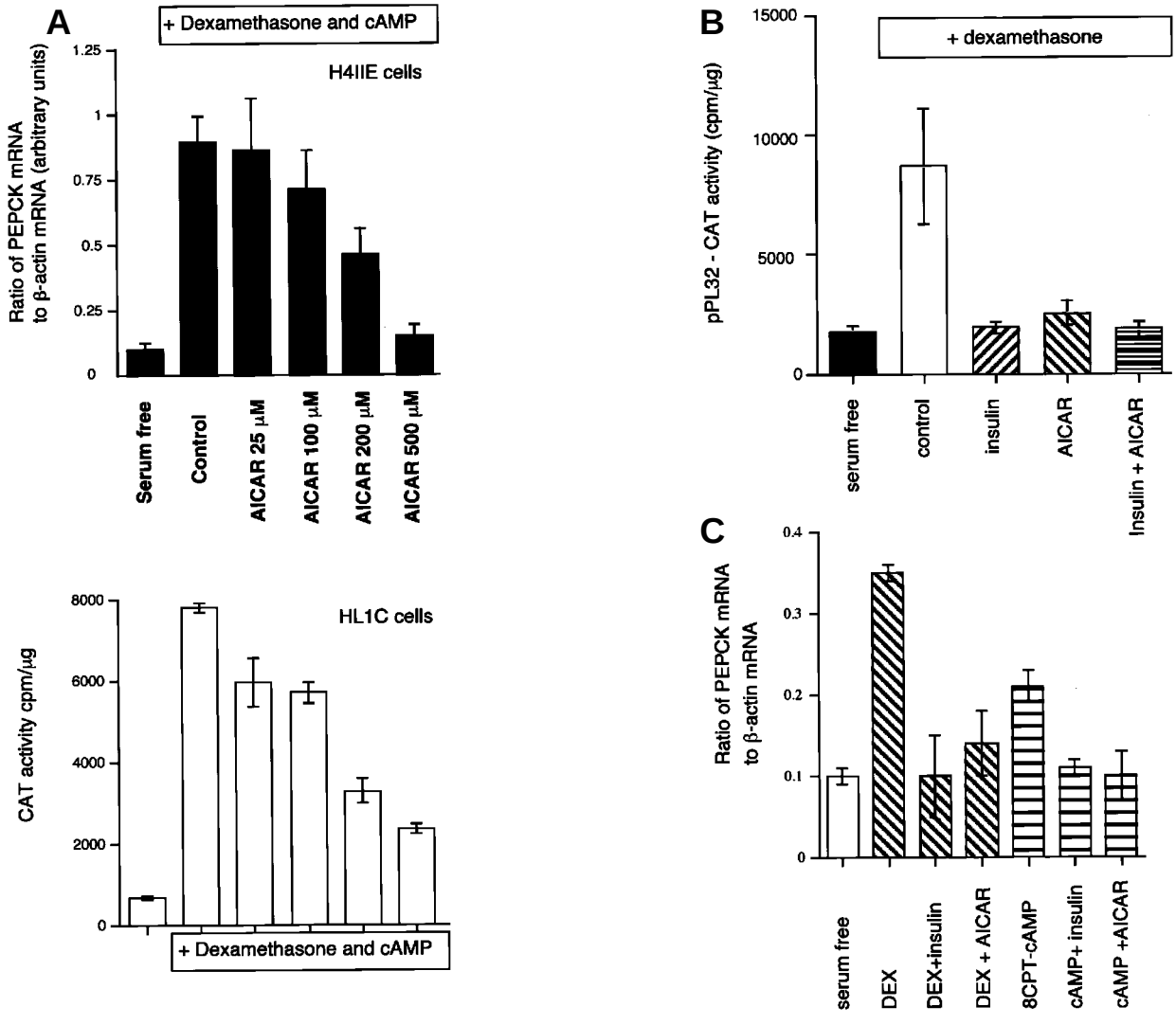

FIG. 1. Treatment of hepatoma cells with AICAR represses PEPCK gene transcription. AICAR repression of PEPCK gene transcription is dose dependent and represses endogenous mRNA as well as stably and transiently transfected PEPCK promoter-CAT reporter expression. Cells were serum starved overnight before a 3-h incubation with hormones as indicated. The concentrations of hormones used were as follows: dexamethasone, 500 nmol/l; 8CPT-cAMP, 0.1 mmol/l; insulin, 10 nmol/l; and AICAR, as indicated. *A*: Primer extension analysis of PEPCK mRNA after treatment with dexamethasone plus 8CPT-cAMP in the presence or absence of insulin or a range of concentrations of AICAR (upper panel). A similar experiment measuring CAT expression under the control of the PEPCK promoter in HL1C cells (a stably transfected clone of the rat hepatoma cell line H4IIE [lower panel]) demonstrates that the effects of AICAR are at the level of the PEPCK promoter. Results are the average ± SE of 3 experiments carried out in duplicate. *B*: Effects of AICAR (500 μmol/l) on induction of CAT expression by dexamethasone after transient transfection of a PEPCK promoter-CAT reporter expression construct (pPL32). Hormone concentrations are as above. Results are the average ± SE of 3 experiments carried out in triplicate. *C*: Repression of both dexamethasone- (DEX) and 8CPT-cAMP–induced endogenous PEPCK promoter activity is obtained with 500 μmol/l AICAR. Results are the average ± SE of 3 experiments carried out in duplicate. Quantitation of PEPCK and β-actin mRNA (by primer extension) and CAT activity is detailed in RESEARCH DESIGN AND METHODS.

500 μmol/l (Fig. 1***A***). The same effect is seen on dexamethasone- and cAMP-induced CAT expression in a stably transfected cell line termed HL1C (Fig. 1***A***). This cell line contains the PEPCK promoter sequence from –2100 to 69 ligated to the CAT reporter gene (35). AICAR also blocks dexamethasone-induced expression of a PEPCK promoter-CAT reporter gene (pPL32) in transient transfection assays (Fig. 1***B***). Finally, the induction of PEPCK gene transcription by glucocorticoids alone (Fig. 1***B*** and ***C***), 8CPT-cAMP alone (Fig. 1***C***), or the 2 in combination (Fig. 1***A***) is repressed by AICAR. There is no effect of AICAR on β-actin (Fig. 1) or cyclophilin mRNA (Fig. 2), indicating that the effects are promoter specific.

G6Pase represents a second important gluconeogenic enzyme and also catalyzes the last step in glycogenolysis (14). Induced and basal hepatic G6Pase gene transcription is almost completely inhibited by insulin administration (Fig. 2). AICAR partially mimics these actions of insulin (Fig. 2), with 30% repression of induced and 50% repression of basal G6Pase gene transcription obtained in the presence of 500 μmol/l AICAR (Fig. 2).

The transcription of the G6PDHase gene is increased by insulin in hepatocytes (40) or H4IIE cells (Fig. 3), albeit by only 1.5- to 2-fold. However, in contrast to PEPCK and G6Pase gene expression, AICAR treatment of H4IIE cells for 3 h (data not shown) or 20 h (Fig. 3), at concentrations up to

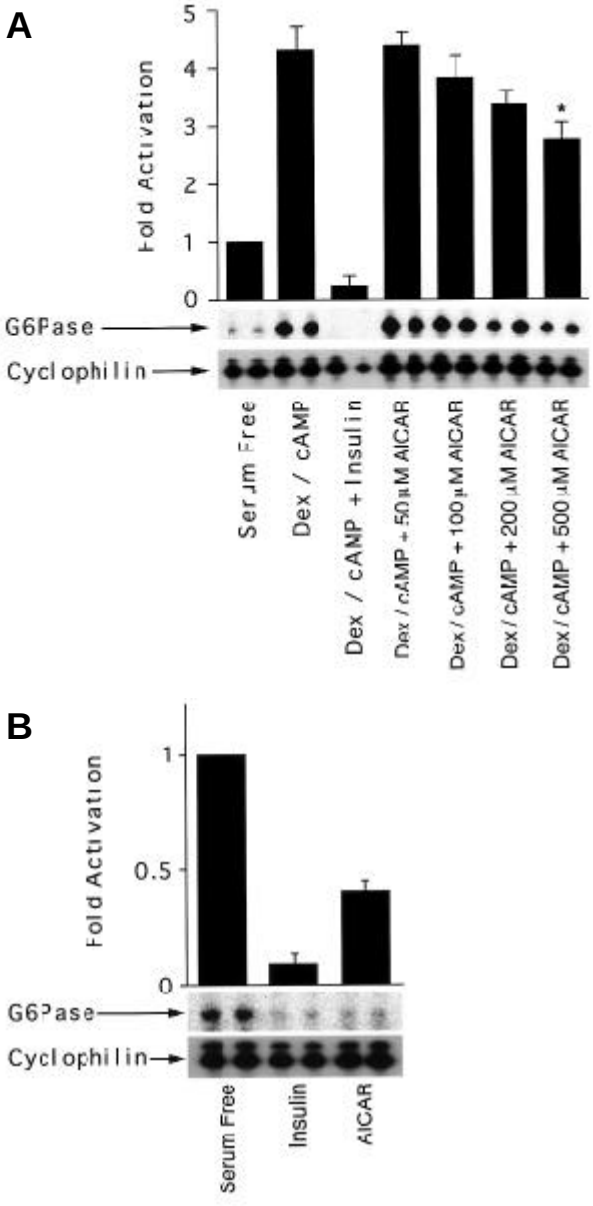

FIG. 2. AICAR partially represses induced and basal G6Pase gene expression. H4IIE cells were serum starved overnight before a 3-h incubation with hormones as indicated. Total RNA was isolated, and an RNase protection assay was performed as described in RESEARCH DESIGN ANDMETHODS. The concentrations of hormones used were as follows: dexamethasone, 500 nmol/l; 8CPT-cAMP, 0.1 mmol/l; (Dex/cAMP) and insulin, 10 nmol/l; and AICAR as indicated. *A*: AICAR partially reduces G6Pase gene expression induced by glucocorticoids and cAMP; the lower panel is a representative experiment, whereas the average ± SE of 4 such experiments is shown in the upper panel (*$P < 0.05$ compared with untreated control). *B*: AICAR (500 µmol/l) reduces basal G6Pase gene expression; the lower panel is a representative experiment, whereas the average ± SE of 4 such experiments is shown in the upper panel.

500 µmol/l, has no effect on basal expression of this gene again demonstrating that repression of PEPCK and G6Pase gene expression is a promoter-selective action of AICAR.

The effects of AICAR on gene transcription are likely to be mediated through activation of AMPK. In H4IIE cells, AICAR activates AMPK at concentrations similar to those required for regulation of PEPCK or G6Pase gene transcription (Fig. 4***A***). The major subunit isoforms of AMPK found in

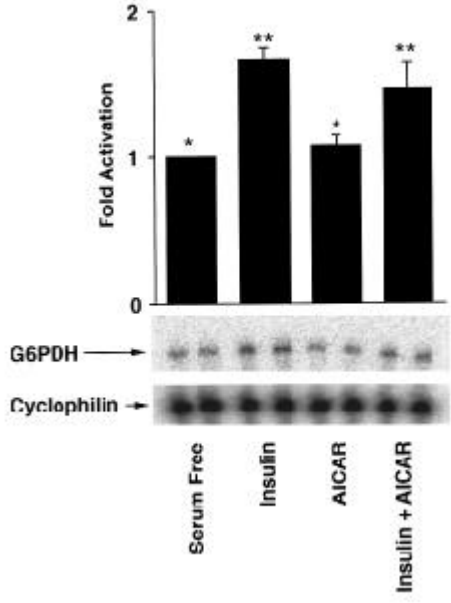

FIG. 3. AICAR has no effect on the expression of G6PDHase. H4IIE cells were serum starved overnight before a 22-h incubation with hormones as indicated. Total RNA was isolated, and an RNase protection assay was performed as described in RESEARCHDESIGNANDMETHODS. The lower panel is a representative experiment, whereas the average ± SE of 4 such experiments is shown in the upper panel. *,NS.**

these cells are α1, β1, and γ1 (data not shown), although these cells contain a higher proportion of AMPK activity contributed by the α2-subunit (27.4% of total α-subunit) than other liver-derived cell lines that we have examined (e.g., CCL13 cells). Approximately 55% of AMPK activity in primary rat hepatocytes is contributed by the α2 isoform (41). The addition of sodium arsenite to H4IIE cells activates AMPK (Fig. 4***B***) and also results in repression of G6Pase (Fig. 4***C***) and PEPCK (23) gene transcription. The effects of arsenite on gene expression are seen at concentrations similar to those that activate AMPK (Fig. 4) (23). Thus, activation of AMPK by 2 distinct reagents correlates with the repression of PEPCK and G6Pase gene transcription observed in these cells. However, although AICAR produces a rapid and sustained induction of AMPK in these cells, there is no significant effect of insulin on the activity of this protein kinase (Fig. 5) at insulin concentrations that result in full repression of PEPCK and G6Pase gene transcription (10 nmol/l). Thus, AMPK itself is not a mediator of insulin-regulated gene transcription in hepatoma cells.

DISCUSSION

PEPCK and G6Pase are 2 key hepatic gluconeogenic enzymes whose expression and activity are increased in type 2 diabetes and in animal models of diabetes (10–15). The increase in the rate of gluconeogenesis contributes to increased hepatic glucose output. The higher level of expression of these genes appears to result from a loss of insulin sensitivity in the tissue, since this hormone strongly and dominantly represses the expression of both genes in healthy liver. The molecular mechanism invoked by insulin to repress these genes is not clear, although recent attention has focused on a DNA sequence (TG/ATTTT/GG/T) common to both gene promoters (42) that is part of the insulin response sequence (IRS) of each promoter. The G6Pase gene pro-

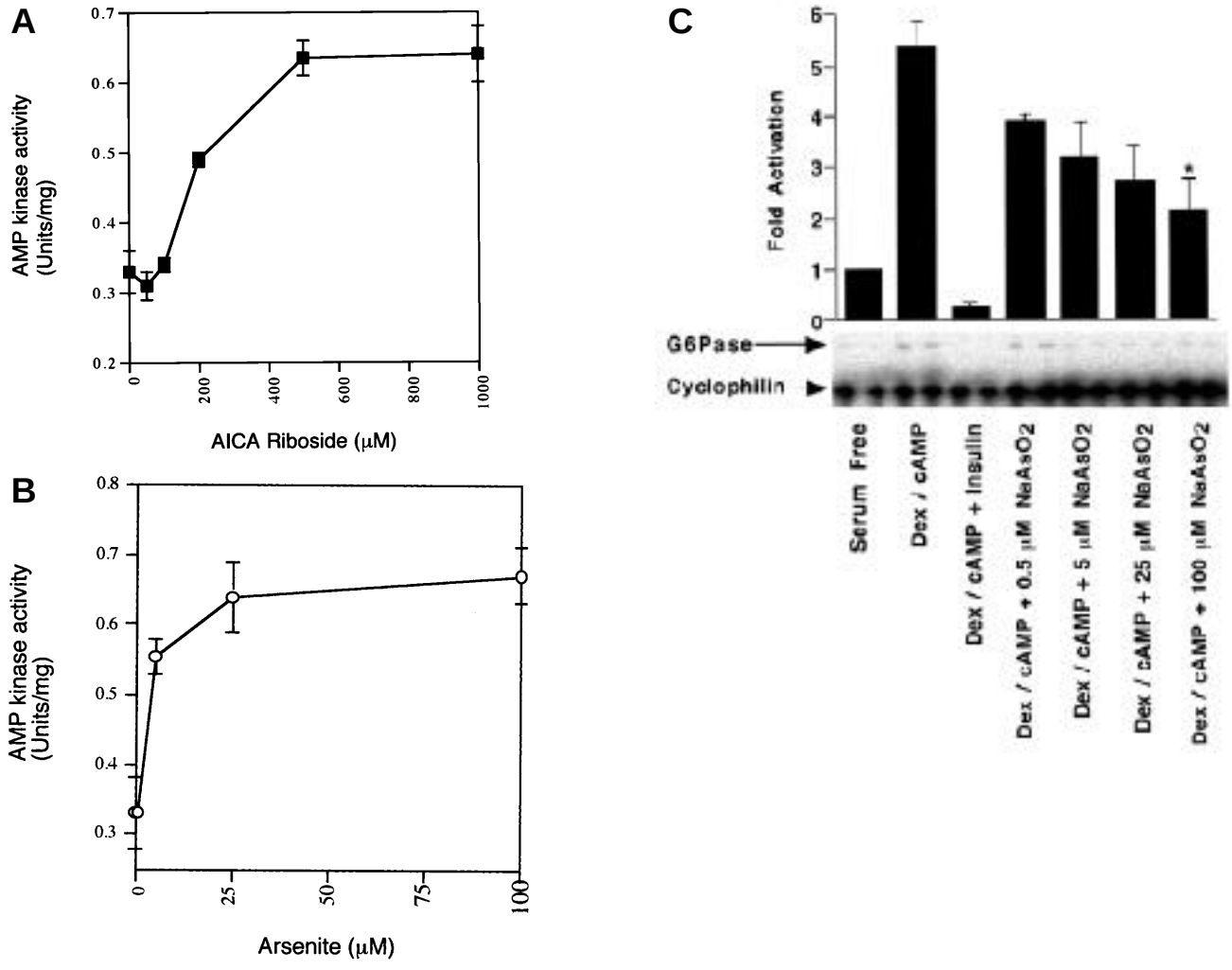

FIG. 4. The effects of AICAR and sodium arsenite on gene expression correlate with activation of AMPK. H4IIE cells were serum starved overnight before a 5-min incubation with AICAR (*A*) or a 3-h incubation with sodium arsenite (*B* and *C*) at the concentrations indicated. Cell lysates were prepared and the activity of AMPK was measured (*A* and *B*), or total RNA was isolated and G6Pase mRNA was determined (*C*) as described in RESEARCH DESIGN AND METHODS. Results are the average ± SE of 2 experiments carried out in duplicate (P* < 0.01).**

moter contains 3 such sequences, and the PEPCK gene promoter contains 1. Because the hormonal regulation of these 2 genes is very similar, and since this sequence is found in the IRS of 2 other gene promoters whose activity is reduced by insulin (namely IGF binding protein 1 [43,44] and tyrosine aminotransferase [45,46]), it has been proposed that a similar mechanism may be involved in the repression of all of these genes by insulin. However, this sequence is also found in numerous gene promoters whose activity is not affected by insulin (42). In addition, the transcription of the PEPCK gene is repressed by glucose (25), whereas that of the G6Pase promoter is induced by glucose (47,48). Recently, troglitazone, an antidiabetic agent that reduces blood glucose and triglyceride levels (49,50), was shown to repress PEPCK gene expression without affecting G6Pase gene expression (6). We demonstrate here that although both gene promoters are repressed by AICAR and sodium arsenite, the transcription of the PEPCK promoter is much more sensitive to either agent. The repression of PEPCK expression by AMPK activation is almost complete, yet induction of G6Pase expression by glucocorticoids and cAMP is only partially repressed after AMPK activation. All of these data suggest that, despite the existence of a short common sequence of DNA in each promoter, there are likely to be different elements and/or mechanisms for repressing the transcription of each gene. Indeed, the difference in sensitivity of each promoter suggests that the target of the AICAR-induced pathway is a key dominant regulator of PEPCK gene expression while (assuming that the AMPK target is the same for both genes) playing only a small part in the repression of G6Pase expression. This again suggests that insulin requires an additional (or distinct) mechanism to fully repress G6Pase expression but would not require an additional mechanism for repression of PEPCK.

We have previously reported that sodium arsenite can dominantly repress PEPCK gene transcription (23). We now show that this agent also partially reduces G6Pase gene expression (Fig. 4***C***). It is known that arsenite treatment of certain cell types raises the intracellular AMP:ATP ratio and thus activates AMPK (29). Arsenite treatment of H4IIE cells activates AMPK to a level equivalent to that obtained by AICAR treatment (Fig. 4). Thus, we have used this agent as an additional approach to obtain evidence for AMPK activation leading to regulation of these gene promoters. Although arsenite reduces the intracellular ATP concentration, this

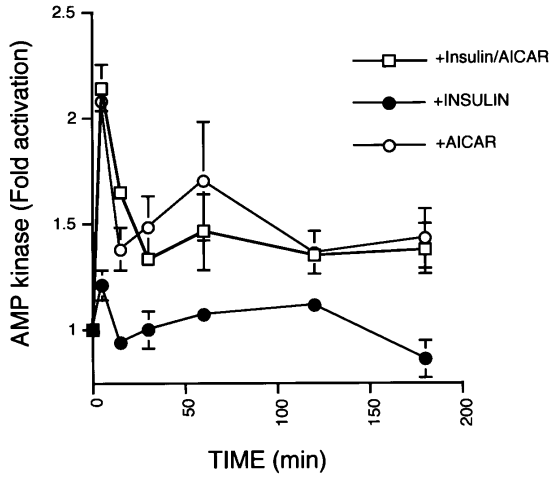

FIG. 5. Insulin does not activate AMPK in H4IIE cells. H4IIE cells were serum starved overnight before addition of insulin (10 nmol/l) and/or AICAR (500 µmol/l) for the times indicated. Cell lysates were prepared, and the activity of AMPK was measured as described in RESEARCHDESIGNAND METHODS. Results are the average ± SE of 2 experiments carried out in duplicate.

should not result in a general reduction of protein kinase activity and lead to the loss of cAMP-dependent protein kinase induction of PEPCK or G6Pase gene expression. Indeed, our data (Fig. 4***B***) demonstrate that arsenite treatment activates AMPK in H4IIE cells, a process that occurs because of phosphorylation at Thr-172. In addition, this agent is well documented to increase the activity of stress-activated protein kinases, such as p38 MAP kinase, a process that requires active phosphorylation within the cell. Importantly, there is a strong correlation between the concentrations of arsenite required for activation of AMPK and the effect of arsenite on gene expression (Fig. 4) (23); thus, AMPK represents a potential link between sodium arsenite and the repression of PEPCK and G6Pase gene promoters. Similarly, the concentrations of AICAR required to activate AMPK match the concentrations of this agent that affect gene expression. There is currently a lack of a selective inhibitor or strong dominant-negative mutant of AMPK (despite a great effort to obtain one) to conclusively address this point. However, the data presented are consistent with the effects of AICAR and arsenite both being mediated by AMPK activation.

Although there are reports that insulin causes a slight inhibition of AMPK activity in FAO hepatoma cells (51) and heart (52), we could not detect a significant change in the activity of this kinase after treatment of H4IIE cells with 10 nmol/l insulin (Fig. 5). By contrast, AICAR (or arsenite) causes a >2-fold activation of AMPK within 5 min. This is followed by a slight decrease in activity, but the activity is then maintained at 50% above the control for up to 3 h (Fig. 5). The regulation of AMPK is complex (27), involving allosteric activation and phosphorylation at Thr-172 by an upstream kinase (53), both of which are stimulated by AMP (54). It is worth pointing out that only the effect of phosphorylation can be observed in a cell extract, the allosteric effect being lost when the cells are homogenized. The true degree of activation of AMPK by arsenite and AICAR in the cells may be greater than is apparent in Fig. 5. We cannot rule out the possibility that regulation of AMPK by insulin could occur by a mechanism other than phosphorylation.

AICAR has numerous rapid effects on metabolism, including the following: inhibition of fatty acid, triglyceride, and sterol biosynthesis in hepatocytes (33,34,55); inhibition of fatty acid synthesis and lipolysis in adipocytes (33,56); and activation of fatty acid oxidation in skeletal muscle and hepatocytes (57,58). In these cases, the effects are known to be due to direct phosphorylation of metabolic enzymes (26). AICAR has also been reported to ***1***) stimulate glucose uptake and GLUT4 translocation in skeletal and cardiac muscle (57,59–62); ***2***) inhibit the expression of fatty acid synthase, pyruvate kinase, and spot 14 genes in hepatocytes (63,64); ***3***) modulate insulin release in pancreatic β-cells (32); ***4***) inhibit autophagy in hepatocytes (65); and ***5***) protect against apoptosis in thymocytes (66) and fibroblasts (67). Although the latter effects are also assumed to be due to activation of AMPK, the protein targets that are phosphorylated are not known. Similarly, the AMPK substrate or substrates that mediate the repression of PEPCK or G6Pase gene expression are not known, although in yeast, the transcription factor Mig1p is a substrate for the SNF1 complex (68). Mig1p is related to the mammalian Egr/Krox-20 and Wilms' tumor transcription factor (69), but the similarity lies only within the sequence of the zinc finger domain, and it is not clear whether there is a true Mig1p homolog in mammals.

We considered whether AICAR could act by antagonism of either the glucocorticoid or cAMP signaling cascades. However, the full effect of AICAR is obtained whether PEPCK gene transcription is induced by glucocorticoid or 8CPT-cAMP, alone or in tandem (Fig. 1). In addition, AICAR reduces basal G6Pase gene expression by 50% (Fig. 2) and has effects on glucose-induced expression of fatty acid synthase, pyruvate kinase, and spot 14 in hepatocytes (63,64). Thus, it is more likely that the effect of AICAR is a direct repression of each promoter, independent of the stimulus. This also suggests a direct and possibly widespread mechanism for gene repression by AMPK and predicts a role for AMPK in the regulation of expression of genes of lipid metabolism as well as gluconeogenesis (28).

Our results show that insulin administration and AMPK activation have similar effects on the expression of PEPCK. Insulin appears to have no effect on AMPK activity; thus, AMPK may not lie directly downstream in an insulin-signaling pathway. Instead, these 2 agents may activate distinct pathways or pathways that converge at a point downstream of AMPK, to affect gene expression. It will be of great interest to observe whether investigation of the pathway downstream of AMPK produces clues as to how insulin regulates these promoters.

Intriguingly, the finding that AMPK activation causes a dramatic inhibition of PEPCK expression in H4IIE cells suggests that activation of this kinase cascade would inhibit gluconeogenesis in vivo. In fact, AICAR has already been shown to inhibit gluconeogenesis in isolated rat hepatocytes (70), as well as hepatic glucose output in mice (71) and rats (60). The phosphorylated form of the riboside, ZMP, mimics the inhibitory effect of AMP on hepatic fructose-1,6-bisphosphatase (F16BPase) as well as the stimulatory effect on AMPK, probably due to direct inhibition of F16BPase. It is possible that an increase in intracellular AMP acutely inhibits gluconeogenesis by inhibiting F16BPase, whereas a more chronic effect of AMP on PEPCK gene expression would prolong the reduced gluconeogenic rate once AMP levels

normalized. Further studies are required to tease out the relative importance of F16BPase inhibition and AMPK activation in vivo. This may require development of a method that activates AMPK but does not inhibit F16BPase.

In summary, AMPK activation appears to mimic 2 key actions of insulin, by which it reduces blood glucose, i.e., stimulating glucose uptake by muscle and inhibiting gluconeogenesis in liver. Because the effects of activation of AMPK appear to be independent of insulin, our findings reinforce the view (28) that pharmacological activation of AMPK might be an effective method to reduce hyperglycemia in type 2 diabetes. It also lends some support to the speculation (28) that some cases of type 2 diabetes might be caused by a low activation state of the AMPK system in the first place.

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