Inhibition of glucokinase translocation by AMP-activated protein kinase is associated with phosphorylation of both GKRP and 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase

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Mukhtar MH, Payne VA, Arden C, Harbottle A, Khan S, Lange AJ, Agius L. Inhibition of glucokinase translocation by AMP-activated protein kinase is associated with phosphorylation of both GKRP and 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase. Am J Physiol Regul Integr Comp Physiol 294: R766–R774, 2008. First published January 16, 2008; doi:10.1152/ajpregu.00593.2007.—The rate of glucose phosphorylation in hepatocytes is determined by the subcellular location of glucokinase and by its association with its regulatory protein (GKRP) in the nucleus. Elevated glucose concentrations and precursors of fructose 1-phosphate (e.g., sorbitol) cause dissociation of glucokinase from GKRP and translocation to the cytoplasm. In this study, we investigated the counter-regulation of substrate-induced translocation by AICAR (5-aminoimidazole-4-carboxamide-1-beta-D-ribofuranoside), which is metabolized by hepatocytes to an AMP analog, and causes activation of AMP-activated protein kinase (AMPK) and depletions of ATP. During incubation of hepatocytes with 25 mM glucose, AICAR concentrations below 200 μM activated AMPK without depleting ATP and inhibited glucose phosphorylation and glucokinase translocation with half-maximal effect at 100–140 μM. Glucose phosphorylation and glucokinase translocation correlated inversely with AMPK activity. AICAR also counteracted translocation induced by a glucokinase activator and partially counteracted translocation by sorbitol. However, AICAR did not block the reversal of translocation (from cytoplasm to nucleus) after substrate withdrawal. Inhibition of glucose-induced translocation by AICAR was greater than inhibition by glucagon and was associated with phosphorylation of both GKRP and the cytoplasmic glucokine binding protein, 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (PFK2) on ser-32. Expression of a kinase-active PFK2 variant lacking serine-32 partially reversed the inhibition of translocation by AICAR. Phosphorylation of GKRP by AMPK partially counteracted its inhibitory effect on glucokinase activity, suggesting altered interaction of glucokinase and GKRP. In summary, mechanisms downstream of AMPK activation, involving phosphorylation of 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase and GKRP are involved in the ATP-independent inhibition of glucose-induced glucokinase translocation by AICAR in hepatocytes.

THE RATE OF GLUCOSE PHOSPHORYLATION in hepatocytes is determined by glucokinase (GK; hexokinase IV) and its regulatory protein (GKRP) that functions as both an inhibitor and nuclear binding protein for GK (2, 11, 34). Binding of GK to GKRP is counteracted by fructose 1-P, which causes translocation of GK to the cytoplasm with a consequent increase in glucose phosphorylation (4, 34). Elevated glucose concentrations and pharmacological activators of GK that bind to an allosteric site on the enzyme also cause translocation of GK to the cytoplasm (4, 10).

Various binding proteins of GK have been identified that could act as cytoplasmic binding proteins. They include a dual-specificity protein phosphatase (27) and the bifunctional enzyme PFK2 (7), which catalyzes the formation and degradation of fructose 2,6-P2 (21) and functions as a cytoplasmic binding protein for GK (32). The liver isoform of PFK2 is regulated by phosphorylation of ser-32 by cAMP-dependent kinase (23), which decreases the kinase/bisphosphatase activity ratio, resulting in depletion of fructose 2,6-P2 (21). Glucagon counteracts both the increase in fructose 2,6-P2 caused by elevated glucose and the translocation of GK from the nucleus. These effects are reversed by the expression of a kinase-active PFK2 variant lacking serine-32 (31), as a result of sequestration of GK in the cytoplasm by the dephosphorylated form of PFK2 (32).

Various compounds and metabolic conditions inhibit substrate-induced translocation of GK from the nucleus to the cytoplasm by mechanisms that cannot be explained by activation of cAMP-dependent protein kinase (1, 2, 19, 29). They include cyanide, which depletes ATP (3); ethanol and glycerol, which promote a more reduced cytoplasmic NADH/NAD state (2, 29); resorcinol, which has similar metabolic actions as the antihyperglycemic drug proglycosyn (1); and AICAR and metformin (19), which are activators of AMP-activated protein kinase (AMPK) and also deplete ATP (20, 42). Selective activators of AMPK are currently considered to be a potential therapeutic strategy for type 2 diabetes (25, 28). However, AICAR inhibits hepatic glucose uptake and stimulates hepatic glycogenolysis (12, 33). It is, therefore, important to understand the mechanisms by which it affects hepatic glucose metabolism.

The aim of this study was to test whether the inhibition of glucose phosphorylation by AICAR (36) can be explained by a mechanism involving activation of AMPK.

MATERIALS AND METHODS

Materials. AMP-activated protein kinase (rat liver) was obtained from Upstate Biotechnology (Lake Placid, NY). GK activator (GKA), 6-[(3-
isobutoxy-5-isopropoxybenzoyl)anilinojicotinic acid (10) and recombi-
nant rat liver GK and rat GKRP (10) were gifts from K. Brocklehurst at
AstraZeneca. SAMS peptide (HMRSMASGLHLVKR) and GKRP-
peptides [QKFQRELTWKVLN, KFQRELS(P)TKWVLNC] were syn-
thesized by Dr. G. Bloomberg, University of Bristol.

Hepatocyte isolation and culture. Hepatocytes were isolated from
male Wistar rats or from male outbred Bkl:BKW-BKW mice (ob-
tained from B&K, Hull, UK) by collagenase perfusion of the liver
(31). Experiments were carried out in accordance with EC Council
directive (86/609/EEC). Hepatocytes were suspended in MEM con-
taining 7% newborn calf serum and cultured in multiwell plates or on
gelatin-coated coverslips for immunostaining. After cell attachment
(2–4 h), the medium was replaced by serum-free MEM containing
5 mM glucose and 10 nM dexamethasone. For experiments with
protein overexpression, 2 h was allowed for cell attachment, and the
monolayers were then incubated for 2 h with the adenoviral vectors
[Ad-PPK2, Ad-PPK2-M (6), Ad-LGK (8), and Ad-GKRP (14)]. Ex-
periments were performed after 16-h culture in MEM containing 5 mM
glucose and 10 nM dexamethasone.

Hepatocyte immunostaining. Glucose phosphorylation and glycolysis were determined from 1 or 3 h
incubations in medium with 25 mM glucose and either [2-3H]glucose or [3-3H]glucose (0.5 μCi/ml) (14). Fructose 2,6-P2 was
determined as described previously (31), and ATP and glucose 6-P were
determined fluorimetrically (9).

Enzyme activity determination. GK activity (free and bound) was
assessed as described in the study by Agius et al. (5), and free activity is
expressed as % total activity. AMP-activated protein kinase was
determined by a modification of the method used by Witters and
Kemp (40). Hepatocyte monolayers were washed once in saline and
then permeabilized for 8 min in lysis buffer containing 250 mM
succrose, 50 mM NaF, 50 mM NaCl, 50 mM Na2PO4, 20 mM Tris pH
7.4, 0.1 mM vanadate, 2 mM ATP, 4 mM MgCl2, 1 mM NAD, 1 mM DTT, 0.05%
phenylmethylsulfonyl fluoride, and 0.1 mg/ml digitonin. The
digitonin eluate was centrifuged (10 min, 13,000 g), and the supernatant
was precipitated with 0.54 volume of saturated (NH4)2SO4 by incu-
bation on ice for 30 min and sedimented at 10,000 g for 20 min. The
pellet was resuspended in 200 μM AMP, 50 mM HEPES, 50 mM
NaCl, 5 mM MgCl2. The reaction was started by addition of 32P-ATP (200 μM ATP, 1 μCi per
assay) and stopped by spotting onto P81 papers (2 × 2 cm),
which were washed 6 times in 1% phosphoric acid, and the radioactivity was
determined.

GK immunostaining. Coverslips were rinsed in PBS and fixed in
4% paraformaldehyde in PBS (30 min). Treatment with NaBH4,
preblocking, and staining with a rabbit IgG against human GK
residues 318-405 (Santa Cruz Biotechnology, Santa Cruz, CA) and
FITC-labeled anti-rabbit IgG were described previously (10). Imaging
was performed using a Nikon Eclipse E400 epifluorescence micro-
scope and Nikon DXM1200 digital camera. Three representative
fields were selected for each condition comprising between 50 and 90
nuclei. The mean pixel intensity of the nuclei and cytoplasmic areas
were determined, and the results were expressed as
nuclei/cytoplasmic ratio. Expression of results as a ratio corrects
for drifts in fluorescence intensity.

32P-labeling of proteins in hepatocytes. The hepatocytes were washed 3 times with saline and incubated for 20 min in phosphate-free
MEM (Invitrogen, Carlsbad, CA). The medium was then replaced by
phosphate-free MEM-containing [32P]phosphate (0.25 μCi/ml), and
the additions were indicated. After 2 h, the medium was removed, and
the monolayers were washed twice with saline and extracted in 800 μl
of lysis buffer (50 mM HEPES, 240 mM sodium deoxycholate,
0.1% SDS, 1% NP-40, 1 mM Na orthovanadate, 10 mM Na-glycer-
ophsphate, 50 μM Na-pyrophosphate, 50 mM NaF, 0.1% β-mer-
captoprotoenol, 50 mM calcium A, 100 mM okadaic acid, 1 mM PMSF,
2.5 μg/ml leupeptin, 1 mM benzamidine, pH 7.4). The lysates were
left on ice for 30 min and sedimented at 10,000 g (10 min). The
supernatants were incubated with 10 μl of either rabbit polyclonal
IgG against GK (H-88, 200 μg/ml; Santa Cruz) or goat polyclonal IgG
against GKRP (N-19; Santa Cruz). After 2 h, 20 μl protein A/G
agarose beads (Calbiochem, San Diego, CA) were added and
incubated overnight at 4°C. The beads were sedimented at 400 g for 10 min
and washed twice with lysis buffer supplemented with 0.5 M NaCl
and a further 2 times with lysis buffer without salt. The beads were
resuspended in 20 μl of loading buffer and separated on SDS-PAGE
(8% SDS). Radioactivity was determined after SDS-PAGE (8% SDS)
fractionation, transfer to nitrocellulose, and phosphorimaging.

32P-labeling of proteins and peptides in vitro. 32P-labeling of
proteins and peptides was performed by a modification of the method
of Warner et al. (39). The assay medium contained 20 mM HEPES, 10
mM MgCl2, 2 mM DTT, 200 μM ATP, 10 μM calcium A, 32P-ATP
(0.25 Ci/mM), AMPK (0.5 units/ml) without or with 200 μM AMP,
and the concentration of peptide indicated or 0.1 mg/ml protein.
Assays were performed at 30°C for 30 to 120 min in a final volume of
20 μl. For assays on synthetic peptides, the incubation was stopped
by spotting on to P81 paper as described above for the AMPK assays.
Protein labeling was determined by phosphorimaging after SDS-
PAGE.

Inhibition of GK by GKRP. The effect of AMPK-induced phos-
phorylation of GKRP on its activity was determined by preincubating
rat recombinant GKRP (10 μg) in a final volume of 90 μl containing
20 mM HEPES, 10 mM MgCl2, 2.5 mM ATP, and 1 U AMPK (as
indicated) for 20 min at 30°C. GKRP activity was then determined by
a modification of a technique used by Brocklehurst et al. (10) in a final
assay mixture containing 5 μg/ml GKRP, 50 mM HEPES, 50 mM
KiCl, 2 mM ATP, 4 mM MgCl2, 1 mM NAD, 1 mM DTT, 0.05% wt/vol BSA, 5 mM glucose, pH 6.9, 15 mM MgCl2 recombinant rat liver
GK and the concentrations of sorbitol 6-P as indicated. GK activity in
the presence of GKRP (5 μg/ml) was expressed as a percentage of
activity in the absence of GKRP.

Immunoblotting. Proteins were fractionated by SDS-PAGE and
transferred to nitrocellulose. Membranes were probed for PKF2,
(P-ser-32) as in (31), and immunoreactive bands were detected using
an enhanced chemiluminescence kit.

RESULTS

AICAR inhibits glucose phosphorylation by an ATP-independent mechanism. AICAR is metabolized by phosphorylation to
5-aminomidazole-4-carboxamide 1-beta-p-ribofuranotide (ZMP) (38), which because of its structural similarity to AMP,
interacts with enzymes with an AMP allosteric site such as
AMPK, fructose 1,6-bisphosphatase, and glycogen phosphor-
ylase. AICAR has been widely used as an activator of AMPK
(13, 20). However, at high concentrations, it causes ATP
depletion (36), and accordingly, its validity as an AMPK
activator is limited to concentrations that do not lower ATP.
We tested the effects of AICAR concentration on AMPK
activation, cell ATP, and glucose metabolism in hepatocytes
(Fig. 1). AICAR caused significant lowering of ATP at 500
μM, and of glucose 6-P at 200 μM (Fig. 1A and B). The
AICAR concentration that caused half-maximal activation of
AMPK was 100 ± 6 μM at 15 min and 136 ± 3 μM at 30 min
(Fig. 1C). The activation reached a peak at 15 to 30 min and
was sustained at 60 min (Fig. 1D). The concentrations of
AICAR that caused half-maximal inhibition of glucose phos-
phorylation and glycolysis were 98 ± 1 and 66 ± 8 μM,
respectively (Fig. 1E). At AICAR concentrations that did not

lower ATP (50–200 μM), glucose phosphorylation correlated inversely with AMPK activity (Fig. 1F, r = 0.993, P = 0.008).

Inhibition of glucose phosphorylation correlates with GK translocation. Incubation of hepatocytes with 25 mM glucose causes dissociation of bound GK and translocation from the nucleus to the cytoplasm (3, 4). AICAR concentrations of 100 μM and above significantly counteracted the effects of 25 mM glucose on GK translocation and dissociation (Fig. 2A and B), with half-maximal effect at 79 ± 5 μM (Fig. 2B). At AICAR concentrations that did not lower ATP (50–200 μM), glucose phosphorylation correlated with GK translocation (Fig. 2C, r = 0.998, P = 0.003), and GK translocation (like glucose phosphorylation, Fig. 1F) correlated inversely with AMPK (r = 0.99, P = 0.02, not shown).

Effects of AICAR on translocation induced by a GK activator and sorbitol. Dissociation of GK from GKRPs and translocation of GK from the nucleus to the cytoplasm is induced not only by elevated glucose but also by pharmacological activators of GK, which bind to an allosteric site (10) and by precursors of fructose 1-P, which decreases the affinity of GKRPs for GK (34, 35). We therefore tested the effects of AICAR on dissociation of bound GK induced by a GKA (10).
This effect is unlikely to be due to inhibition by AICAR of formation of fructose 1-P at the level of ketohexokinase because AICAR did not prevent the decline in cellular ATP caused by 5 mM fructose (control 11.0 ± 1.5; fructose 5.2 ± 0.9; AICAR 12.0 ± 1.9; AICAR + fructose 1.6 ± 0.6 nmol/mg protein). We also tested the effects of 200 μM AICAR on GK translocation in mouse hepatocytes. AICAR suppressed GK dissociation (P < 0.01) after 60 min incubation with 25 mM glucose or with 10 μM GKA or 200 μM sorbitol (free GK % total: control, 32 ± 3; 25 mM glucose 44 ± 3; GKA, 48 ± 3; sorbitol, 46 ± 3; AICAR, 24 ± 2; 25 mM glucose + AICAR, 30 ± 2; GKA + AICAR, 37 ± 2; sorbitol + AICAR 39 ± 2) and as in rat hepatocytes did not lower cellular ATP.

Comparison on glucose-induced and sorbitol-induced GK translocation. Translocation of GK caused by precursors of fructose 1-P is more rapid than translocation by glucose (3), indicating involvement of different mechanisms. We therefore tested the effects of AICAR (200 μM) on the time course of GK translocation induced by either sorbitol or 25 mM glucose. AICAR did not counteract the initial rapid rate of GK translocation after sorbitol addition (2–5 min) but partially counteracted the effect at later time points. In contrast, AICAR counteracted the effect of glucose at all time points (Fig. 3, A and B).

We tested whether AICAR (200 μM) affects the movement of GK from the cytoplasm to the nucleus by preincubation of hepatocytes with 25 mM glucose and 200 μM sorbitol followed by incubation in medium with 5 mM glucose. AICAR did not counteract the movement of GK from the cytoplasm to the nucleus (Fig. 3C).

Effect of metformin. We tested the effects of metformin, which, like AICAR, inhibits glucose phosphorylation in hepatocytes. Effects of metformin (0.1–20 mM) on AMPK activity, cell ATP, and glucose metabolism were determined as described above for AICAR. At 0.5 and 5 mM, metformin stimulated AMPK (control, 1.6 ± 0.4; 0.5 mM, 4.6 ± 1.4; 5 mM, 12.7 ± 1.4 pmol·min⁻¹·mg⁻¹ means ± SE, n = 7; P < 0.005), lowered ATP (control, 7.5 ± 0.4; 0.5 mM, 6.3 ± 0.6; P < 0.005; 5 mM, 4.2 ± 0.6 nmol/mg; P < 0.005) and inhibited glucose-induced GK translocation (GK N/C: control, 2.2 ± 0.2; 0.5 mM, 2.8 ± 0.3; P < 0.005; 5 mM, 3.0 ± 0.4; P < 0.005). Significant activation of AMPK occurred only at concentrations that caused ATP depletion. Thus metformin could not be used to investigate mechanisms downstream of AMPK that are independent of ATP depletion.

Role of fructose 2,6-P₂ and PFK2. Hormones or compounds that lower fructose 2,6-P₂ in hepatocytes, such as glucagon, ethanol, and resorcinol (1, 2, 29, 31) inhibit GK translocation. Because AICAR also lowers fructose 2,6-P₂ (36), we tested whether expression of a kinase active (bisphosphatase deficient) mutant of PFK2 lacking ser-32 (PFK2-M) reverses the effects of AICAR on GK translocation. AICAR caused a smaller suppression of fructose 2,6-P₂ than glucagon (P < 0.005) but a greater suppression of glucokinase translocation (P < 0.05, Fig. 4). Expression of PFK2-M by eight-fold relative to endogenous activity caused more than twofold increase (P < 0.005) in fructose 2,6-P₂ (Fig. 4A) and reversed the effects of glucagon and ethanol on translocation but partially reversed the inhibition by AICAR (Fig. 4B).

or by sorbitol, a precursor of fructose 1-P during a 1-h incubation at 5 mM glucose. AICAR (200 μM) partially counteracted the dissociation of GK caused by the GKA (10 μM) or by 200 μM sorbitol (free GK % total: control, 31 ± 5; GKA, 50 ± 5; sorbitol, 46 ± 6; AICAR, 32 ± 4; AICAR + GKA, 38 ± 5*; AICAR + sorbitol, 40 ± 5*, means ± SE, n = 4, *P < 0.05 relative to no AICAR). AICAR also suppressed but did not abolish the stimulation of glucose phosphorylation caused by 200 μM sorbitol at 5 mM glucose (control 382 ± 58; sorbitol 503 ± 68; AICAR 141 ± 21; AICAR + sorbitol 313 ± 12 nmol·3 h⁻¹·mg protein⁻¹, means ± SE; n = 3).

Fig. 2. Effects of AICAR concentration on glucokinase (GK) translocation. A and B: hepatocytes were incubated in MEM with 5 mM glucose and the concentrations of AICAR indicated for 30 min before the addition of 20 mM glucose. Incubations were stopped after 60 min for determination of GK nuclear/cytoplasmic ratio (A) and free and bound glucokinase activity (B). C: glucose phosphorylation against free GK at 0–200 μM (open symbols) or 500 μM (solid symbol) AICAR. Values are means ± SE for 4 experiments. *P < 0.05; **P < 0.005 relative to no AICAR at 25 mM glucose.
Role of protein phosphorylation in mediating the effects of AICAR. Since glucagon lowers fructose 2,6-P$_2$ in liver by phosphorylation of PFK2 on ser-32 (23), we next tested whether the suppression of fructose 2,6-P$_2$ by AICAR is also associated with phosphorylation of ser-32. For these experiments, hepatocytes were either untreated or treated with an adenoviral vector to overexpress wild-type PFK2. AICAR increased the phosphorylation of both endogenous and overexpressed PFK2 on ser-32 (Fig. 5). The increase in phosphorylation by AICAR was less than that caused by glucagon (Fig. 5). We also tested for effects of AICAR on phosphorylation of GK and GKRP by prelabeling hepatocytes with $^{32}$P-inorganic phosphate, followed by immunoprecipitation of GK and GKRP. In these experiments, GK and GKRP were overexpressed by between two- and four-fold using adenoviral vectors. $^{32}$P-labeling was detected in the GKRP immunoprecipitates at 55–70 kDa only very weakly at 35 kDa in the GK immunoprecipitates of AICAR-treated cells (Fig. 6). Labeling of recombinant human-GKRP and human-GK with $^{32}$P-ATP showed variable incorporation of label in different preparations of recombinant protein up to less than 0.3 mol/mol (results not shown). The GKRP-peptide QKFQRELSTKWVLN (474-487) was phosphorylated by purified AMPK with twofold lower affinity than the SAMS peptide (Fig. 7A: apparent $K_m$: GKRP-peptide, 101 ± 24 μM; SAMS, 55 ± 8 μM). Extracts

Fig. 3. Effects of AICAR on the time course of glucokinase translocation after substrate addition or removal. A and B: hepatocytes were preincubated in MEM with 5 mM glucose without (open symbols) or with (solid symbols) 200 μM AICAR before the addition of either 200 μM sorbitol (A) or 20 mmol/l glucose (B). Incubations were terminated at the time indicated after substrate addition for determination of glucokinase nuclear/cytoplasmic ratio. C: hepatocytes were preincubated for 30 min with 25 mM glucose and 200 μM sorbitol in either the absence (open symbols) or presence (solid symbols) of 200 μM AICAR. The medium was then replaced by medium containing 5 mM glucose without or with AICAR, and incubations terminated at the time intervals indicated. Incubations maintained with 5 mM glucose are also shown in the left-hand bar. Values are means ± SE for 4 experiments. *P < 0.05 relative to no AICAR (A–C); #P < 0.05; ##P < 0.005 relative to time zero (C).
interacted with GK. This suggests that phosphorylation of GKRP by AMPK modifies the interaction of GKRP with GK.

In hepatocytes incubated with AICAR increased phosphorylation of the GKRP peptide four-fold compared with six-fold for the SAMS peptide (Fig. 7B), indicating that the GKRP peptide is a potential substrate for AMPK and/or other kinases activated by AICAR in hepatocytes.

We tested whether incubation of recombinant GKRP with AMPK and ATP in conditions associated with phosphorylation (as above) affects the inhibitory activity of GKRP on glucokinase (Fig. 7C). Preincubation of GKRP with either ATP/Mg2+ or with AMPK alone had no effect on the inhibition of glucokinase activity by GKRP. However, preincubation of GKRP with AMPK plus ATP/Mg2+ caused a significant ($P < 0.01$) increase in GK activity (decrease in inhibition by GKRP) in both the absence of sorbitol 6-P (GK activity % control: 80 ± 2 to 85 ± 2, means ± SE, n = 5) and in the presence of 250 μM sorbitol 6-P (35 ± 2 to 48 ± 3) relative to the incubations with ATP/Mg2+ alone, and there was no difference in the EC50 for sorbitol 6-P (13 ± 2 vs. 15 ± 3 μM). This suggests that phosphorylation of GKRP by AMPK modifies the interaction of GKRP with GK.

**DISCUSSION**

AICAR has been widely used as an activator of AMPK (13, 20). Unlike the expression of a constitutively active AMPK variant (16, 17), which is a model of chronic activation, AICAR enables the study of acute activation of AMPK. However, its limitations are that at high concentrations, it causes depletion of ATP due to sequestration of phosphate as ZMP (37) and also in addition to activating AMPK, ZMP also affects other enzymes with AMP allosteric sites (20, 38). Activation of AMPK by AICAR results in inhibition of fatty acid synthesis by phosphorylation (inactivation) of acetyl CoA carboxylase, and the consequent suppression of the product malonyl-CoA causes stimulation of fatty acid oxidation (18). In skeletal muscle and heart, activation of AMPK by AICAR or anoxia is associated with stimulation of glucose uptake and glycolysis (24, 25). In liver, in contrast, intraportal infusion of AICAR causes complete suppression of hepatic glucose uptake and stimulation of glycogenolysis in both hyperinsulinemic and hypoglycemic conditions (12, 33). Skeletal muscle and heart express different isoforms of PFK2 from liver. Skeletal muscle expresses a variant of PFKFB1 that lacks the N-terminal 32 amino acid residues and unlike the liver isoform is not regulated by cAMP-dependent protein kinase. The heart isoform (PFKFB2) is activated by AMPK by phosphorylation of ser-466 (24).

The effects of AICAR on hepatic glucose metabolism were first reported by Vincent and colleagues (36). They showed suppression of gluconeogenesis, which was explained by ZMP-mediated inhibition of fructose 1, 6-bisphosphatase through its AMP allosteric site (37) and inhibition of glucose phosphorylation by a mechanism that could not be fully explained by a direct effect of ZMP or AICAR on GK (36). This raised the question of whether activation of AMPK by AICAR could explain the inhibition of glucose phosphorylation. The recent study by Guigas et al. (19) tested this possibility in hepatocytes from transgenic mice lacking both alpha-1 and alpha-2 AMPK.
catalytic subunit. They showed that 1 mM AICAR inhibited glucose phosphorylation and glucokinase translocation in hepatocytes from both wild-type mice and AMPK-deficient mice. At this AICAR concentration, there was significant depletion of ATP in hepatocytes from wild-type mice (by −50%) and more extensive ATP depletion (by ~80%) in hepatocytes from AMPK-deficient mice, suggesting that the latter are more prone to ATP depletion, presumably because of the lack of feedback mechanisms activated by AMPK. In these conditions AICAR inhibited glucokinase translocation as a result of ATP depletion.

In this study, we show that AICAR concentrations in the range of 50–200 μM, which cause activation of AMPK in the absence of ATP depletion, inhibit glucose-induced glucokinase translocation in rat and mouse hepatocytes. The inverse correlation between AMPK activity and both glucose phosphorylation and glucokinase translocation suggests that AICAR inhibits glucose phosphorylation by an ATP-independent mechanism downstream of ZMP formation and/or AMPK activation through inhibition of glucokinase translocation. Although metformin also inhibited glucokinase translocation, it caused significant ATP depletion at all concentrations that were associated with activation of AMPK. It was therefore not possible to distinguish between a role for AMPK as distinct from ATP depletion with metformin. This is consistent with previous studies that showed that a primary site of action of metformin is the mitochondrial complex I (rather than AMPK) (15, 30). Accordingly, activation of AMPK by metformin (42) is most likely secondary to ATP depletion by inhibition of the respiratory chain (15). Because AICAR caused half-maximal activation of AMPK at ~100 μM and it caused ATP depletion at concentrations above 200 μM, it can be inferred that AICAR (unlike metformin) can be used for in vitro studies to test mechanisms downstream of activation of AMPK that are independent of ATP depletion.

Glucose and precursors of fructose 1-P have synergistic effects on GK translocation (4). The effect of precursors of fructose 1-P is more rapid than the effect of glucose (3) and is explained by fructose 1-P-induced dissociation of GK from GKRP (29, 34). The effect of glucose is mimicked by nonmetabolizable glucose analogs (4) and by pharmacological GKAs (10). In this study, AICAR counteracted translocation induced by both glucose and the GKA, and it also partially counteracted translocation induced by sorbitol, a precursor of fructose 1-P. However, in the latter case, inhibition was more marked at later rather than early time points after sorbitol addition. This suggests that rather than inhibition of the initial rate of nuclear export after sorbitol addition, AICAR may favor the shuttling of GK back to the nucleus. There are no known selective inhibitors of nuclear export or import of GK (26), and measurement of net changes in nuclear to cytoplasm distribution of GK (in the absence of selective inhibitors) does not distinguish between changes in nuclear export as opposed to import. If there is constitutive nuclear-cytoplasmic shuttling of either GK or the GK-GKRP complex, then the counteraction by AICAR of substrate-induced translocation from the nucleus to the cytoplasm could be due to inhibition of nuclear export, stimulation of nuclear import, inhibition of dissociation of the GK-GKRP complex or increased dissociation of GK from cytoplasmic receptors. The present results, which demonstrate I an increase in the nuclear/cytoplasmic ratio of GK in various
metabolic conditions; 2) lack of effect on the initial rate of sorbitol-induced translocation; and 3) lack of effect on the rate of reversal of translocation, support a mechanism involving decreased binding of GK to cytoplasmic receptors during nuclear-cytoplasmic shuttling. In addition, the decreased inhibition of GK by GKRP after phosphorylation by AMPK is suggestive of a change in interaction of GK with GKRP.

Various binding proteins of GK have been identified that may function as cytoplasmic receptors (7, 27). The bifunctional enzyme PFK2 binds to GK through its bisphosphatase domain (7). A role for the liver isoform of PFK2 in sequestering GK in the cytoplasm is supported by studies on the mechanism by which glucagon affects glucokinase translocation (31, 32). Glucagon causes phosphorylation of PFK2 on ser-32, resulting in depletion of fructose 2,6-P2 and translocation of glucokinase from the cytoplasm to the nucleus. These effects of glucagon are reversed by expression of a kinase-active mutant of PFK2 lacking ser-32 (31, 32). Guigas and colleagues showed that AMPK causes dual phosphorylation of liver PFK2 on ser-22 and on either ser-32 or ser-33 (19). In this study, we show that AICAR causes phosphorylation of PFK2 on ser-32. We also show that AICAR caused a greater inhibition of glucose-induced translocation than glucagon and that the effect of AICAR was partially but not fully counteracted by expression of PFK2-M lacking ser-32 (31, 32). Guigas and colleagues showed that AMPK causes dual phosphorylation of liver PFK2 on ser-22 and on either ser-32 or ser-33 (19). In this study, we show that AICAR causes phosphorylation of PFK2 on ser-32. We also show that AICAR caused a greater inhibition of glucose-induced translocation than glucagon and that the effect of AICAR was partially but not fully counteracted by expression of PFK2-M lacking ser-32, whereas the effect of glucagon was fully counteracted. It is possible that the dual phosphorylation of PFK2 on ser-22 and ser-32 may account for the greater inhibition of translocation by AICAR compared with glucagon and also for the lack of total reversal of translocation by expression of PFK2-M (S32A,H258A).

Phosphorylation of GKRP by AMPK is an alternative explanation for the greater inhibition of glucokinase translocation by AICAR compared with glucagon. A GKRP peptide (476-485) with an AMPK consensus motif (20) that is conserved in human and Xenopus GKRP, which share 88% and 57% identity, respectively, with rat GKRP (35, 39) as a substrate for AMPK and for kinases that are activated by AICAR in hepatocytes (Fig. 7). The finding that AMPK counteracted the inhibitory activity of GKRP on GK was surprising. Since activation of AMPK in hepatocytes was associated with increased sequestration of glucokinase in the nucleus, one might have expected increased rather than decreased inhibition of glucokinase by GKRP after phosphorylation with AMPK. It is possible that phosphorylation of GKRP by AMPK results in an altered conformation of the GK-GKRP complex with increased GK activity rather than decreased binding of GK to GKRP. A change in conformation of the GK-GKRP complex could affect the binding of the dimeric complex to either nuclear import or nuclear export proteins that transport cargo across the nuclear pore complex or to macromolecules within the nucleus and thereby favor nuclear sequestration.

Perspectives and Significance

The subcellular location of GK within the hepatocyte has a major role in the control of hepatic glucose metabolism, as shown by the very high control strength of GK on glucose metabolism, particularly in conditions in which the enzyme was largely sequestered in the nucleus (5). Rapid translocation of glucokinase from the nucleus to the cytoplasm is induced by an increase in glucose concentration and by precursors of fructose 1- P, as well as by pharmacological GKAs (3, 10). We show in this study that micromolar concentrations of AICAR, which cause activation of AMPK without depletions of cellular ATP, counteract substrate-induced GK translocation by a mechanism that is associated with phosphorylation of both GKRP and PFK2, which are the nuclear and cytoplasmic binding proteins of GK, respectively. In these conditions, AICAR is a more potent inhibitor of GK translocation than glucagon, which inhibits translocation by phosphorylation of PFK2 on ser-32 (31, 32). Thus phosphorylation of both the nuclear and the cytoplasmic binding proteins of GK by AMPK is an important regulatory mechanism counteracting substrate-induced GK translocation.

The inhibition of hepatic glucose metabolism by AICAR contrasts with the stimulation of glucose uptake and glycolysis in skeletal muscle and heart, which express different isoforms of PFK2 (24, 25). This can be reconciled by the different roles of glycolysis in liver and muscle. In muscle the function is to provide ATP, and accordingly, it is regulated by energy charge, which signals ATP demand. However, in the liver, the main function of glycolysis is to convert dietary carbohydrates to lipids, and it is regulated by substrate supply and feed-forward activation. It can be concluded that AMPK inhibits hepatic lipogenesis through multisite control, involving inhibition of GK translocation, with consequent inhibition of flux through glucose phosphorylation and glycolysis; phosphorylation and inactivation of acetyl-CoA carboxylase; phosphorylation and inactivation of transcription factors involved in regulation of lipogenic genes (22); and counteraction of expression of lipogenic genes by glucose 6-P generated by glucokinase (17, 41). The latter can now also be explained, at least in part, by inhibition of GK translocation.

REFERENCES


