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Inhibition of Hepatic Phosphatidylcholine Synthesis by 5-Aminoimidazole-4-carboxamide-1-β-4-ribofuranoside Is Independent of AMP-activated Protein Kinase Activation*

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5-Aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAr), a commonly used indirect activator of AMP-activated protein kinase (AMPK), inhibits phosphatidylcholine (PC) biosynthesis in freshly isolated hepatocytes. In all nucleated mammalian cells, PC is synthesized from choline via the Kennedy (CDP-choline) pathway. The purpose of our study was to provide direct evidence that AMPK regulates phospholipid biosynthesis and to elucidate the mechanism(s) by which AMPK inhibits its hepatic PC synthesis. Incubations of hepatocytes with AICAr resulted in a dose-dependent activation of AMPK and inhibition of PC biosynthesis. Surprisingly, adenoviral delivery of constitutively active AMPK did not alter PC biosynthesis. In addition, expression of dominant negative mutants of AMPK was unable to block the AICAr-dependent inhibition of PC biosynthesis, indicating that AICAr was acting independently of AMPK activation. Determination of aqueous intermediates of the CDP-choline pathway indicated that choline kinase, the first enzyme in the pathway, was inhibited by AICAr administration. Flux through the CDP-choline pathway was directly correlated to the level of intracellular ATP concentrations. Therefore, it is possible that inhibition of PC biosynthesis is another process by which the cell can reduce ATP consumption in times of energetic stress. However, unlike cholesterol and triacylglycerol biosynthesis, PC production is not regulated by AMPK.

Phosphatidylcholine (PC)[4] is quantitatively the most important phospholipid in mammalian membranes, is the primary phospholipid in bile and in plasma lipoproteins, and is a precursor for the synthesis of sphingomyelin and phosphatidylserine. In nucleated cells, PC biosynthesis occurs via the Kennedy (CDP-choline) pathway. Choline kinase (CK), the enzyme catalyzing the first committed step in this pathway, phosphorylates choline to form phosphocholine. Choline kinase is not considered an important site in regulating PC biosynthesis (1–3); however, deletion of the CKβ isoform results in muscular dystrophy in mice due to lack of PC in muscle (4). CTP:phosphocholine cytidylyltransferase (CT), which catalyzes the rate-limiting conversion of phosphocholine to CDP-choline (1–3), is the most extensively studied enzyme in the Kennedy pathway. CT is found in soluble cell and tissue extracts and bound to membranes. The active form of CT in cells is considered to be membrane-associated, whereas the soluble form is thought to be an inactive reservoir (5, 6). Movement of CT to and from the membrane is linked to cell requirements for PC and is tightly regulated (7–14). The lipid binding domain and the highly phosphorylated C terminus of CT are typically involved in regulating its activity. The last enzyme in the pathway, CDP-choline:1,2-diacylglycerol cholinephosphotransferase, is in excess in cells and thus does not determine the rate of PC biosynthesis. This reaction requires the availability of diacylglycerol, which is synthesized from fatty acids and glycerol.

Hepatocytes are unique in that they can also synthesize PC by sequentially methylating phosphatidylethanolamine (PE) via phosphatidylethanolamine *N*-methyltransferase (PEMT). Three *S*-adenosylmethionine molecules are consumed in the course of this reaction (15). PEMT produces 30% of hepatic PC, whereas the CDP-choline pathway accounts for the remaining 70% (16–18). Unlike CT, regulation of PEMT is not clearly defined. PEMT flux is altered by dietary choline and methionine (15, 19, 20), is down-regulated following partial hepatectomy (21), and is induced when CT is deleted (22, 23); however, the molecular mechanism for these changes is unknown.

AMP-activated kinase (AMPK) is a heterotrimeric serine/threonine protein kinase consisting of a catalytic subunit (α) and two regulatory subunits (β and γ) (24). AMPK is believed to be an intracellular energy sensor and is involved in regulating glucose and lipid metabolism (25). In times of ATP

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[4] The abbreviations used are: PC, phosphatidylcholine; CT, CTP:phosphocholine cytidylyltransferase; PE, phosphatidylethanolamine; PEMT, phosphatidylethanolamine *N*-methyltransferase; CK, choline kinase; AICAr, 5-amino-imidazole-4-carboxamide-1-β-D-ribofuranoside; AMPK, AMP-activated protein kinase; ZMP, 5-amino-imidazole-4carboxamide-1-β-D-ribotide; GFP, green fluorescent protein; PBS, phosphate-buffered saline; pfu, plaque-forming units; DN, dominant negative; dpm, disintegrations/min.

depletion, such as hypoxia and glucose deprivation, AMPK activates ATP-generating pathways (*i.e.* lipolysis and glycolysis) while inhibiting anabolic processes, including fatty acid, cholesterol, and triacylglycerol biosynthesis. Under normal conditions, these metabolic changes protect the cell against ATP deprivation. AMPK elicits its action on lipid metabolism by phosphorylating key rate-limiting enzymes, such as acetyl-CoA carboxylase and 3-hydroxy-3-methylglutaryl-coenzyme A reductase (25–27).

5-Aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAr) is a commonly used indirect activator of AMPK (28). AICAr enters cells through the adenosine transporter and is quickly phosphorylated to 5-amino-imidazole-4-carboxamide-1-β-D-ribotide (ZMP). A rise in intracellular ZMP, an analogue of AMP, results in activation of AMPK (29). Recently, AICAr has been shown to inhibit both PE and PC biosynthesis in isolated rat hepatocytes (30). However, this study did not provide a direct link between AMPK activity and phospholipid biosynthesis. Furthermore, the specific site(s) of inhibition remained elusive. Therefore, the purpose of our study was to provide direct evidence that AMPK regulates phospholipid biosynthesis and to elucidate the mechanism(s) by which AMPK inhibits hepatic phospholipid metabolism. In contrast to our hypothesis, we found that although AICAr does indeed inhibit PEMT and choline kinase flux, it does so independently of AMPK activation.

EXPERIMENTAL PROCEDURES

Materials—AICAr was purchased from Toronto Research Chemicals Inc. (Toronto, Canada), and iodotubercidin was obtained from Calbiochem. The green fluorescent protein (GFP) antibody was purchased from Molecular Probes, Inc. (Eugene, OR), and AMPK (derived from the N-terminal human sequence) antibodies were from Cell Signaling Technology (Danvers, MA). The goat anti-rabbit secondary antibodies conjugated to horseradish peroxidase were purchased from Pierce. The polyclonal antibody directed against the C-terminal dodecapeptide of rat PEMT2 was raised in rabbits in our laboratory. All other chemicals and reagents were from standard commercial sources.

Animal Care—All procedures were approved by the University of Alberta's Institutional Animal Care Committee and were in accordance with guidelines of the Canadian Council on Animal Care. Male mice (3–6 months old, C57BL/6;129P2 mixed background) were fed *ad libitum* a chow diet (LabDiet, PICO laboratory Rodent Diet 20) and were exposed to a 12-h light/dark cycle starting at 8:00 a.m.

Primary Hepatocyte Cultures—Primary hepatocytes were isolated by collagenase perfusion and plated on 60-mm collagen-coated dishes at a density of 1.0×10^6 cells/dish in Dulbecco's modified Eagle's medium containing 15% fetal bovine serum. Two h after plating, the cultures were rinsed twice in serum-free Dulbecco's modified Eagle's medium over a 1-h period and then incubated in serum-free Dulbecco's modified Eagle's medium with or without the presence of AICAr for 45 min. Following AICAr preincubation, cells were labeled, in the presence of AICAr, with [^{3}H]ethanolamine, [^{3}H]choline, or [^{3}H]glycerol (1 μCi/ml) for 1 h, after which the medium was aspirated, the cells were washed with 1×2 ml of cold phosphate-buffered saline (PBS), and cells were then scraped into 1 ml of PBS. Homogenates were sonicated for 10 s, and protein was determined by the Bradford method (Bio-Rad) using albumin as a standard.

Fibroblast Cell Culture—C3H10T1/2 mouse embryo fibroblasts were cultured in Dulbecco's modified Eagle's medium supplemented with penicillin G (100 units/ml), streptomycin (100 μg/ml), and 10% fetal bovine serum in a 5% CO_2 humidified incubator at 37 °C. Two days before the experiment, cells were plated on 10-cm dishes and allowed to grow to 70–80%.

Adenoviral Infection of Hepatocytes—To construct recombinant adenovirus, cDNA containing a Myc-tagged constitutively active (T172D) catalytic α_1 subunit (amino acid residues 1–312) of AMPK (CA-AMPK) was subcloned into a pAdTrack-CMV shuttle vector, linearized with PmeI, and inserted into adenovirus using pAdEasy-1 system for homologous recombination in *Escherichia coli*. The pAdTrack-CMV shuttle vector also contained a gene encoding GFP. Therefore, the adenovirus used to express AMPK protein also expressed GFP, which served as a marker of successful viral infection and protein overexpression. A similar protocol was used to construct adenoviruses encoding either a dominant negative mutant of AMPKα1 (DN-AMPKα1) or a dominant negative mutant of AMPKα2 (DN-AMPKα2).

In some experiments, hepatocytes were infected with Ad.GFP, Ad.CA-AMPKα1, or a combination of AD.DN-AMPKα1 and AD.DN-AMPKα2 (25–400 plaque-forming units/cell) for 24 h before pretreatment with AICAr.

Determination of Radiolabeled Products—Lipids were extracted from homogenates using the Folch method (31), and phospholipids were separated by thin layer chromatography (chloroform/methanol/acetic acid/water, 25:15:4:2). Following iodine visualization, bands corresponding to PE and PC were scraped, and radioactivity was measured by liquid scintillation counting. When [^{3}H]glycerol was used, neutral lipids were separated from phospholipids in a second solvent system (diethylether/benzene/ethanol/acetic acid, 45:50:2:0.2).

Radioactivity in water-soluble choline- and ethanolamine-containing compounds was measured in the aqueous phase from the lipid extraction. The aqueous phase was dried under constant air flow. The samples were resuspended in methanol/water (1:1) and loaded onto thin layer chromatography plates with cold carriers, and compounds were separated for 2 h (methanol/0.5% NaCl/NH_4, 10:10:1). Iodine and ninhydrin were used to visualize choline and ethanolamine metabolites, respectively.

Enzymatic Assays—Livers or hepatocytes were homogenized in a mini-Polytron homogenizer in 2 ml of buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM dithiothreitol, and 0.1 mM phenylmethylsulfonyl fluoride) followed by sonication for 30 s. Following centrifugation at 99 K for 15 min, the supernatant (cytosol) was removed, and the membranes were resuspended in homogenization buffer. Four dishes were pooled when cytosolic and membrane fractions were isolated from hepatocytes. Total CT activity was measured in fractions isolated from hepatocytes by monitoring the conversion of [^{3}H]phosphocholine into CDP-choline (32). For measurement of PEMT activity, 50

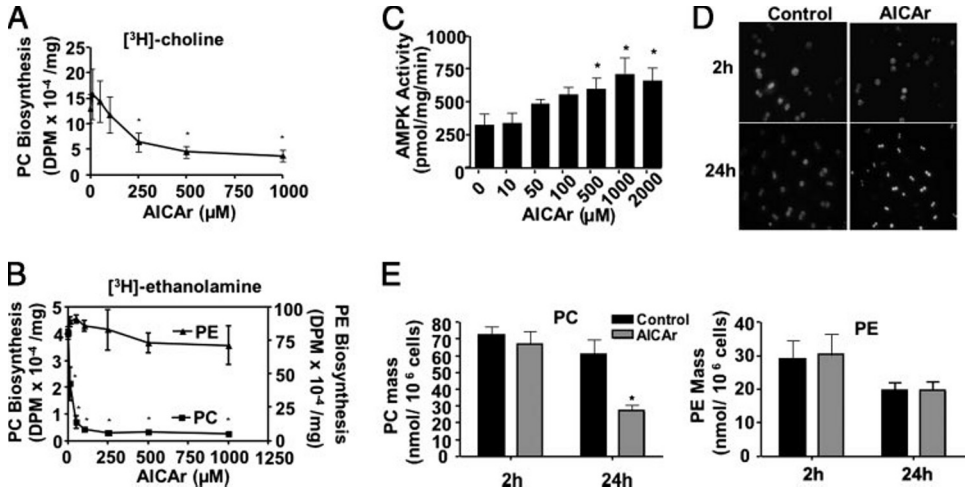

FIGURE 1. **AICAr inhibits incorporation of [^{3}H]choline and [^{3}H]ethanolamine into PC in isolated mouse hepatocytes.** Following a 45-min preincubation with AICAr, hepatocytes were incubated with [^{3}H]choline (*A*) or [^{3}H]ethanolamine (*B*) for 1 h in the presence of AICAR. Cells were collected, and the radiolabel in PE and PC was determined. AMPK activity (*C*) was measured in hepatocytes following AICAr treatment for 105 min. Following a 2- or 24-h AICAR (500 μM) incubation, cells were either stained with Hoechst 33325 (*D*) or homogenized (*E*) for determination of PC and PE. Data are means ± S.D. from three separate hepatocyte preparations. The *asterisks* signify difference *versus* no AICAr treatment, $p < 0.05$.

μg of homogenates was incubated with phosphatidylmonomethylethanolamine and *S*-adenosyl[*methyl*-^{3}H]methionine, and the incorporation of radiolabel was measured as described previously (33). Choline kinase activity was measured as described with minor modifications (34). Cytosols were incubated in a final volume of 100 μl of reaction buffer that contained 0.1 M Tris-HCl, pH 8.75, 2 μM ATP, 15 mM $MgCl_2$, and 0.25 mM [^{3}H]choline chloride (10.5 μCi/ml) at 37 °C for 20 min. The reaction product, phosphocholine, was isolated using an AG1-X8 (200–400 mesh, OH- form) column (Bio-Rad).

For AMPK activity, hepatocytes were homogenized in buffer containing 20 mM Tris·HCl (pH 7.4), 50 mM NaCl, 50 mM NaF, 5 mM sodium pyrophosphate, 0.25 M sucrose, 1% Triton X-100, mammalian protease inhibitor mixture (Sigma), phosphatase inhibitor mixture I (Sigma), and 1 mM dithiothreitol. The sonicated samples were centrifuged at 800 × *g* for 10 min, and the resulting supernatant was isolated. Four μg of the supernatant was assayed for AMPK activity using a peptide substrate as previously described (35).

Immunoblots—Homogenates (5–20 μg of protein) were boiled in buffer containing 1% SDS, and proteins were separated on SDS-polyacrylamide gels. Proteins were transferred to an Immobilion-P transfer membrane and probed with anti-GFP (dilution 1:5000) and anti-AMPKα (dilution 1:1000). Immunoreactive bands were visualized by enhanced chemiluminescence according to the manufacturer's instructions (Amersham Biosciences).

Other Methods—Chromatin condensation was evaluated as a marker for apoptosis. Briefly, cells were washed with PBS (2 × 1 ml) and then fixed with 4% paraformaldehyde for 10 min. Following washing with PBS (3 × 1 ml), hepatocytes were incubated with Hoechst 33325 (1 μg/ml) for 15 min, washed with PBS (3 × 1 ml), and visualized under UV light. The concentration of ATP was measured using a luciferase-based kit purchased from Sigma. Phospholipid mass was measured using a lipid phosphorus assay with sodium phosphate as a standard (36).

Statistical Analysis—Data are presented as means ± S.D. unless otherwise noted. Each experimental group contained three or four samples. Student's unpaired *t* test was performed to compare means unless otherwise specified. A *p* value of <0.05 was considered to be significant.

RESULTS

AICAr Inhibits PC Biosynthesis in Mouse Hepatocytes—PC biosynthesis was inhibited by AICAr treatment in a dose-dependent manner. [^{3}H]choline incorporation into PC was inhibited by 75%, with maximum inhibition reached at 500 μM (Fig. 1*A*). [^{3}H]Ethanolamine incorporation into PE was only decreased by 15% with AICAr treatment; however, [^{3}H]PC formation from PE was inhibited by >90% (Fig. 1*B*). This PEMT-dependent process was completely inhibited by 100 μM AICAr. In agreement with previous reports (37), AICAr treatment increased AMPK activity 2-fold (Fig. 1*C*).

Since inhibition of PC biosynthesis in cultured cells can lead to apoptosis, we assessed the viability of our hepatocytes after AICAr treatment. No difference between treatment groups was observed following the 2-h incubation (Fig. 1*D*). Furthermore, the mass of PC and PE was not altered, suggesting that AICAr does not cause cell damage after a 2-h treatment (Fig. 1*E*). However, after a 24-h incubation, PC levels were decreased by 60% in the presence of AICAR, and almost all hepatocytes displayed signs of apoptosis (Fig. 1, *D* and *E*).

Inhibition of Hepatic PC Biosynthesis Is Not Dependent on AMPK Activation—To assess the role of AMPK in the conversion of [^{3}H]choline into PC, we increased AMPK activity via adenovirus-mediated delivery of a constitutively active mutant of AMPK. After adenoviral delivery, all cells were infected, as assessed by GFP fluorescence (Fig. 2*A*). Expression of the CA-AMPK protein reached maximal at an adenoviral concentration of 200 pfu/cell (Fig. 2*B*). At this adenoviral concentration, total AMPK activity was increased almost 3-fold (Fig. 2*C*). Surprisingly, the increase in AMPK activity did not alter incorporation of [^{3}H]choline or [^{3}H]ethanolamine into PC (Fig. 2, *D* and *E*, respectively), although the increase in AMPK activity was greater following adenoviral delivery than with AICAr treatment. When CA-AMPK-expressing hepatocytes were incubated with AICAr, PC production was again inhibited, although AMPK activity was not further increased (data not shown). In a similar set of experiments, we used adenoviruses containing genes for dominant negative (DN) mutants of AMPKα1 and AMPKα2. Tandem expression of these proteins blocked the AICAr-dependent activation of AMPK (Fig. 3*A*). However, expression of the DN-AMPK mutants did not alter PC biosynthesis and did not prevent the AICAr-dependent

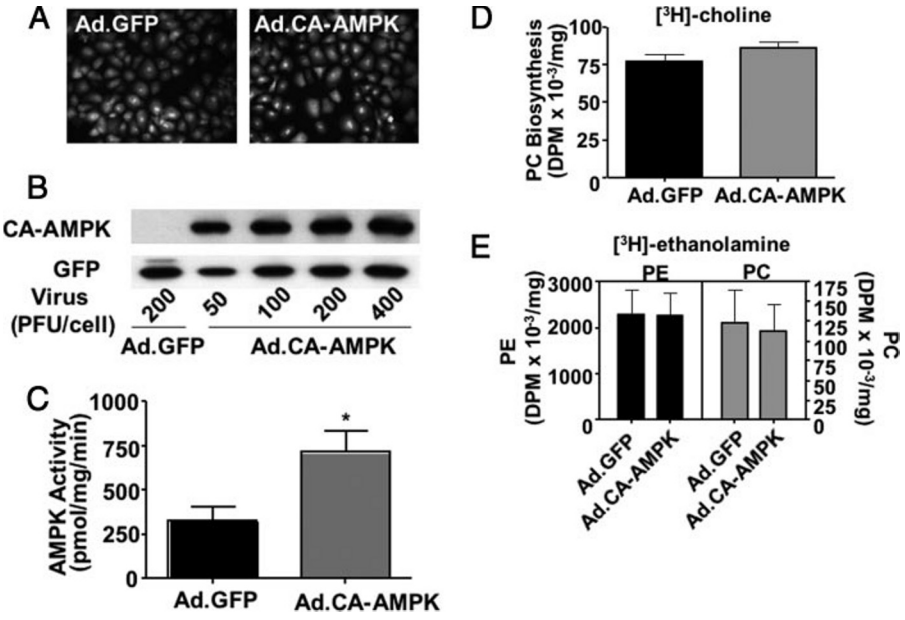

FIGURE 2. **Expression of AMP-activated protein kinase does not inhibit PC biosynthesis in isolated mouse hepatocytes.** Hepatocytes were infected overnight with Ad.GFP or Ad.CA-AMPKα1. *A*, cells were visualized by fluorescent microscopy to determine the proportion of infected cells. *B*, proteins were separated by SDS-polyacrylamide gel electrophoresis, and immunoblotting was performed using antibodies raised against AMPKα1 and GFP. *C*, AMPK activity was measured in the supernatant fraction isolated from hepatocytes that were infected with 200 pfu/cell of Ad.GFP or Ad.CA-AMPKα1. Following overnight infection, hepatocytes were incubated with [^{3}H]choline (*D*) or [^{3}H]ethanolamine (*E*) for 1 h. Cells were collected, and the radiolabel in PE and PC was determined. Data are means $\pm$ S.D. from three separate hepatocyte preparations. The *asterisks* signify difference *versus* no AICAr treatment, $p < 0.05$.

inhibition of PC biosynthesis (Fig. 3, *B* and *C*). Taken together, these results indicate that AICAr inhibits PC biosynthesis independently of AMPK activation.

Mechanism of PC Biosynthesis Inhibition by AICAr—The rate-limiting reaction of the CDP-choline pathway is catalyzed by the enzyme CTP:phosphocholine cytidylyltransferase (1–3). The active form of CT in cells is membrane-bound, whereas the soluble form of CT is thought to be an inactive reservoir. Under normal conditions, the rate of PC biosynthesis is proportional to the amount of membrane-bound CT. Interestingly, AICAr administration resulted in a 3-fold increase in membrane-associated CT activity (Fig. 4*A*), although [^{3}H]choline incorporation into PC was inhibited by 75%. Furthermore, these results indicate that CT is not the location of AICAr action, since increased membrane-bound CT should result in increased PC biosynthesis. To further confirm that CT is not inhibited by AICAr, we incubated hepatocytes with [^{3}H]glycerol to induce [^{3}H]diacylglycerol formation. Consistent with the *in vitro* activity of CT, we observed increased [^{3}H]PC production following AICAr treatment (Fig. 4*B*). Taken together, we conclude that inhibition of [^{3}H]choline incorporation into PC occurs before the reaction catalyzed by CT.

To ascertain the site of inhibition in the CDP-choline pathway, we measured radioactivity in the soluble intermediates. Consistent with a previous report (30), no change in total cellular radioactivity was observed following AICAr treatment (Fig. 5*A*), indicating that choline transport was not impaired. The intracellular level of [^{3}H]choline was also unaltered by treatment (Fig. 5*B*). However, AICAr treatment did result in a dose-dependent decrease in radioactivity found in phosphocholine and CDP-choline, whereas [^{3}H]betaine formation was increased 2-fold (Fig. 5, *C–E*). It is possible that decreased PC production is due to an activation of choline oxidase (the enzyme responsible for converting choline to betaine), an inhibition of choline kinase, or a combination of both. To address the role choline oxidation plays in removing choline from the CDP-choline pathway, we repeated the AICAr experiments in a fibroblast cell line (C3H/10T1/2) that contains minimal choline oxidase activity (38). In these cells, [^{3}H]choline incorporation into phosphocholine and PC was reduced by 70% after AICAr treatment (Fig. 6, *B* and *E*). [^{3}H]Betaine levels were very low in these cells and were not altered by AICAr (Fig. 6*D*). These results confirm that choline oxidase is not required for the reduction in PC biosynthesis. Furthermore, the effects of AICAr are not a hepatocyte-specific phenomenon.

AICAr Treatment Inhibits Choline Kinase in Hepatocytes—Our results thus far suggest that AICAr reduces PC biosynthesis by inhibiting choline kinase independently of AMPK activation. However, following AICAr treatment, choline kinase activity was not altered in cytosols obtained from hepatocytes (control, 0.77 $\pm$ 0.08; 250 μM AICAr, 0.72 $\pm$ 0.06; 1000 μM AICAr, 0.90 $\pm$ 0.09 nmol/mg/min). Therefore, we sought to explain the mechanism of AICAr action within the cell. To this end, we incubated hepatocytes with iodotubercin, an adenosine kinase inhibitor, which prevents phosphorylation of AICAr to ZMP (an intermediate of the *de novo* purine biosynthesis pathway) (Fig. 7*A*). Preincubation with iodotubercin prevented the AICAR-induced inhibition of [^{3}H]choline incorporation into PC (Fig. 7*B*). This suggests that ZMP or one of its downstream metabolites is responsible for inhibiting choline kinase. With this in mind, we investigated whether any of the purine intermediates (Fig. 7*A*) inhibit CK activity *in vitro*. In this experiment, liver cytosolic fractions were incubated with various concentrations of each purine molecule. We found that AICAr, ZMP, IMP, hypoxanthine, inosine, and adenylsuccinate had no effect on choline kinase activity (Fig. 7, *C* and *D*). However, choline kinase activity was inhibited in a dose-dependent fashion by adenosine, AMP, and ADP (Fig. 7*E*). Unlike, CK, *in vitro* CT activity was not altered in the presence of any purine metabolite (Fig. 7*F*). This suggests that the synthesis of phosphocholine by choline kinase is sensitive to the intracellular concentration of ATP, a substrate for CK. To support this hypothesis, we measured the level of ATP in hepatocytes following AICAr treatment. Similar to PC biosynthesis (Fig. 1*A*), there was a slight increase in

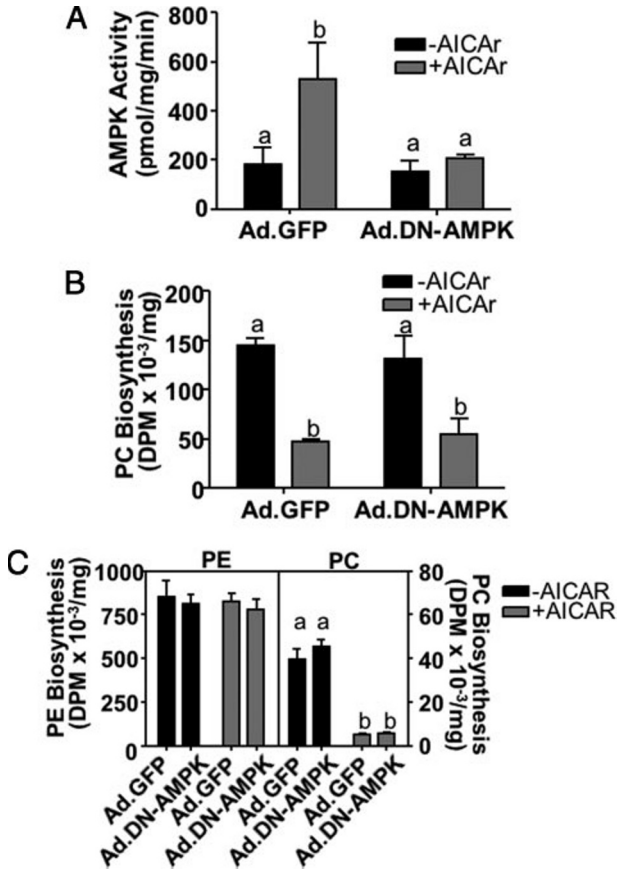

FIGURE 3. **Dominant negative mutants of AMP-activated protein kinase do not prevent the AICAr-induced inhibition of PC biosynthesis.** Hepatocytes were infected overnight with Ad.GFP (100 pfu) or with Ad.DN-AMPKα1 and Ad.DN-AMPKα2 (50 pfu each). *A*, AMPK activity was measured in hepatocytes incubated with or without AICAr (500 μM) for 105 min. Following a 45-min preincubation with AICAr (500 μM), infected hepatocytes were incubated with [^{3}H]choline (*B*) or [^{3}H]ethanolamine (*C*) for 1 h. Cells were collected, and the radiolabel in PE and PC was determined. Data are means $\pm$ S.D. from three separate hepatocyte preparations. Results with *differing letters* are significantly different from each other ($p < 0.05$).

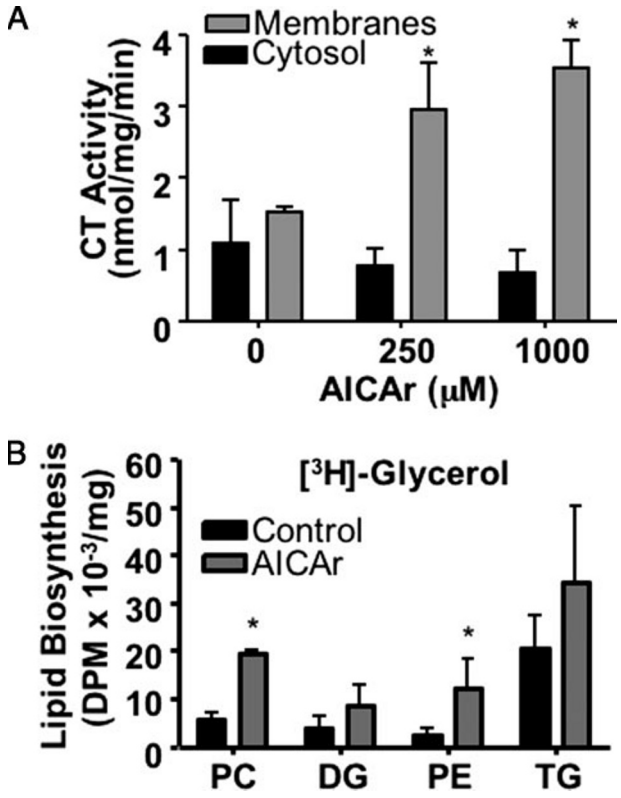

FIGURE 4. **Increased CT translocation and [^{3}H]glycerol incorporation into PC following AICAr treatment.** *A*, membrane and cytosolic fractions from hepatocytes were assayed for CT activity. *B*, following a 45-min preincubation with AICAr (500 μM), hepatocytes were incubated with [^{3}H]glycerol for 1 h in the presence of AICAr. Cells were collected, and the radiolabel in PC, PE, triacylglycerol (*TG*), and diacylglycerol (*DG*) was determined. Data are means $\pm$ S.D. from three separate hepatocyte preparations. The *asterisks* signify difference *versus* no AICAr treatment ($p < 0.05$).

ATP levels when a low level (10 μM) of AICAr was used (Fig. 7*G*). However, increasing concentrations of AICAr led to an 85% decrease in ATP levels (Fig. 7*G*). Furthermore, the level of ATP in the cell directly correlated with production of PC from choline (Fig. 7*H*).

DISCUSSION

In this study, we report that AICAr inhibits PC synthesis by an AMPK-independent mechanism. Although AICAr treatment did activate AMPK activity, several lines of evidence suggest that this enzyme is not involved in regulating PC biosynthesis. First, inhibition of the PEMT and CDP-choline pathway occurred at concentrations of AICAr that did not activate AMPK. Furthermore, the apparent K_i values for both pathways were substantially different (PEMT ~50 μM, CDP-choline ~200 μM), suggesting that different mechanisms were involved in inhibiting these pathways. In addition, adenoviral expression of a constitutively active mutant of AMPK did not influence PC biosynthesis. Finally, dominant negative mutants of AMPK were unable to prevent the AICAR-dependent inhibition of PC biosynthetic.

In hepatocytes, AICAr has been reported to increase AMPK activity without changing the cellular concentrations of adenosine nucleotides (39). Our results cast doubt on this assertion, since, in our hands, ATP levels decreased dose-dependently above 50 μM AICAr. This is consistent with previous reports that concentrations of AICAr above 0.5 mM deplete ATP levels in both hepatocytes and liver (27, 37, 40). Although the exact mechanism is unknown, it has been suggested that AICAr inhibits hepatic oxidative phosphorylation and glycolysis by an AMPK-independent mechanism (37, 41).

Mechanism of Choline Kinase Inhibition by AICAr—Our results indicate that inhibition of choline kinase is responsible for decreased PC biosynthesis through the Kennedy pathway in the presence of AICAr. This was an unlikely finding, since CK does not normally regulate PC biosynthesis in the liver. For example, in CKβ knock-out mice, hepatic CK activity is reduced by 75% (CKα constitutes the remaining activity), yet PC levels remain normal (4). In hearts in CKβ knock-out mice, CK activity was ~1% of normal in knock-out mice; still, PC concentrations were unchanged. CT is considered the rate-limiting enzyme in the CDP-choline pathway, yet in our experiments [^{3}H]choline incorporation into PC was inhibited despite an increase in membrane-bound (active) CT, suggesting that CT is activated in response to CK inhibition. This finding is particularly germane, given that PC mass was unaltered follow-

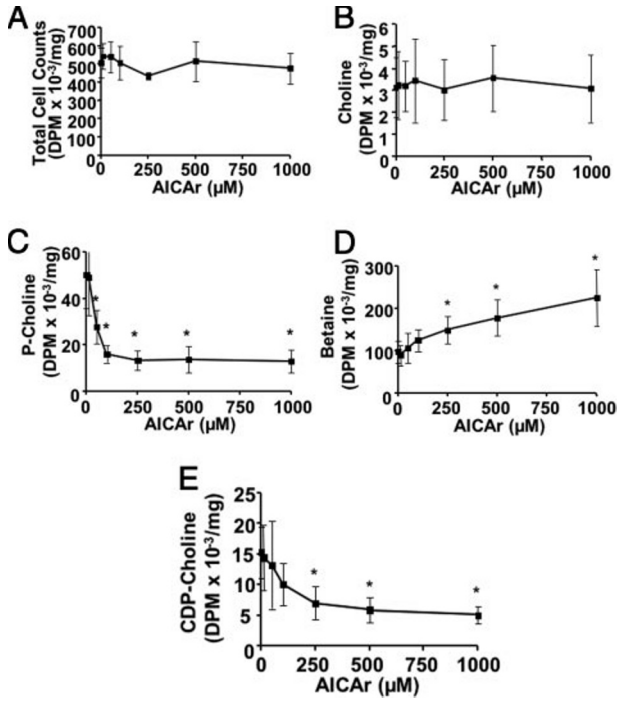

FIGURE 5. **Incorporation of [^{3}H]choline into aqueous intermediates in isolated mouse hepatocytes.** Following a 45-min preincubation with AICAr, hepatocytes were incubated with [^{3}H]choline for 1 h in the presence of AICAr. An aliquot of homogenates was taken to determine total cellular radioactivity (*A*). The radiolabel in choline (*B*), phosphocholine (*C*), betaine (*D*), and CDP-choline (*E*) was determined. Data are means ± S.D. from three separate hepatocyte preparations. The *asterisks* signify difference *versus* no AICAr treatment ($p < 0.05$).

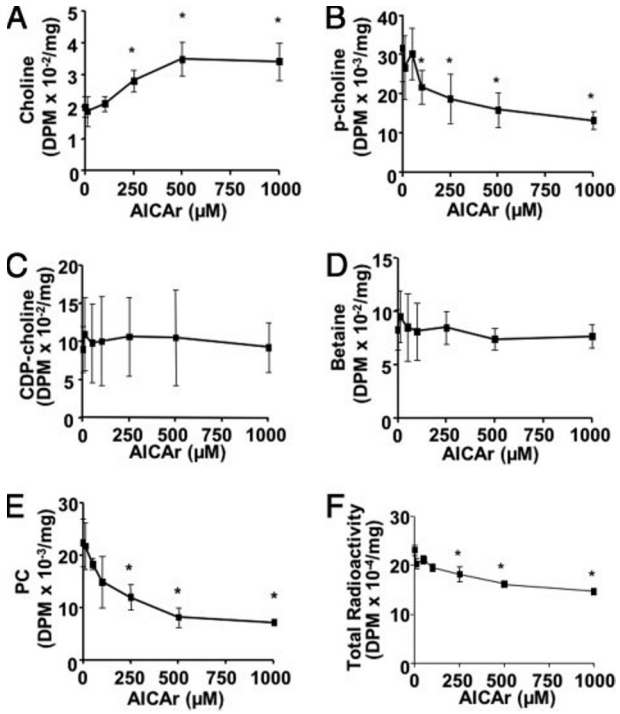

FIGURE 6. **Incorporation of [^{3}H]choline into PC and aqueous intermediates in C3H/10T1/2 cells.** Following a 45-min preincubation with AICAr, cultured fibroblast cells were incubated with [^{3}H]choline for 1 h in the presence of AICAr. Cells were collected, and the radiolabel in choline (*A*), phosphocholine (*B*), CDP-choline (*C*), betaine (*D*), and PC (*E*) was determined. An aliquot of homogenates was taken to determine total cellular radioactivity (*F*). Data are means ± S.D. from three separate hepatocyte preparations. The *asterisks* signify difference *versus* no AICAr treatment ($p < 0.05$).

ing the 2-h AICAr incubation period. It is likely that PC levels were maintained in the cell until the phosphocholine pool was exhausted (Fig. 1*E*). It is unclear what molecular signal stimulated CT translocation in our experiment, although it is known that CT is sensitive to microchanges in membrane lipids and fluidity (14). We have shown that purine metabolites do not alter the *in vitro* activity of membrane-associated CT. Whatever the mechanism, it is clear that in the presence of AICAr, CK becomes rate-limiting in the CDP-choline pathway. It is currently unknown if CT is likewise up-regulated in CKβ knock-out mice where CK flux is inhibited.

We have provided convincing evidence that AICAr inhibits PC biosynthesis by decreasing cellular energy stores. Although the precise mechanism(s) of choline kinase inhibition is unknown, it is possible that either an increase in the ratio of adenosine analogues to ATP or a decrease in ATP levels alone plays a role. The $K_{m(\mathrm{ATP})}$ for choline kinase has been reported to be 3.7 mM, which is similar to the concentration of ATP in isolated hepatocytes (3–5 mM) (42, 43). Therefore, *in vivo* CK activity should be very sensitive to increased or decreased ATP concentrations. Incubations with AICAr provide evidence for both situations. At low concentrations (10 and 50 μM), both ATP and PC biosynthesis were slightly elevated; however, at concentrations above 50 μM, both measurements decreased proportionally. Of course, this does not exclude the role of adenosine, AMP, and ADP in inhibiting CK. It is tempting to suggest that a decrease in ATP must result in an increase in the less phosphorylated adenosine molecules; however, this idea is contrary to the belief that AICAr does not alter adenosine concentrations in the cell. Regardless, if adenosine, AMP, and ADP levels remain constant, the ratio of these molecules to ATP is increased. These molecules act as competitive inhibitors of CK and work at physiological concentrations. These observations may not be limited to experiments involving AICAr. Recently, the biguanide antidiabetic drug, metformin, was shown to reduce ATP in both rat and mouse hepatocytes (37); these authors reported an inhibition of glucokinase that was independent of AMPK activation. Interestingly, we have preliminary results indicating that phenformin, a similar biguanide drug, reduces [^{3}H]choline incorporation into phosphocholine and PC (data not shown), suggesting that CK was inhibited.

AICAr Inhibits Choline Kinase but Not Ethanolamine Kinase—To date, three isoforms of choline kinase termed α1, α2, and β have been reported in the mouse and rat (44). All isoforms also have *in vitro* ethanolamine kinase activity. The specific activity of CK is 4-fold higher when the substrate is choline compared with ethanolamine (42). Moreover, the $K_{m(\mathrm{ethanolamine})}$ is 50 times higher than that for choline (*i.e.* 1.7 mM *versus* 30 μM). Both reactions do appear to occur *in vivo*, since induction of CK results in increased production of both phosphocholine and phosphoethanolamine (45). With this in mind, it was surprising to observe an increase rather than a decrease in synthesis of [^{3}H]phosphoethanolamine from [^{3}H]ethanolamine, in hepatocytes incubated with AICAr (0 *versus* 500 μM AICAr: 10 ± 2 *versus* 21 ± 4 dpm × 10^{-3}/mg of protein, $p < 0.05$). How could

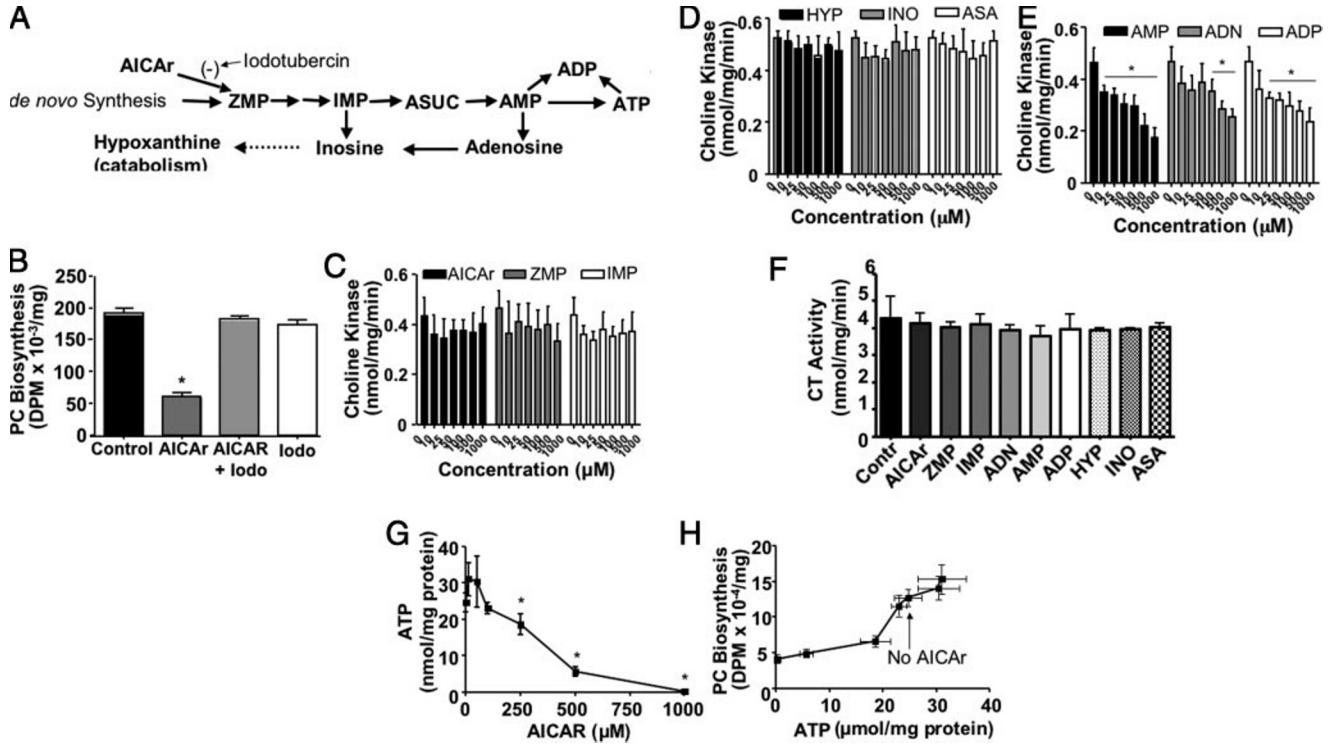

FIGURE 7. **Choline kinase activity is inhibited by alterations in adenosine nucleotides.** *A*, intermediates in the *de novo* synthesis of purine nucleotides. *B*, hepatocytes were treated with or without iodotubercin (*Iodo*) and AICAr (500 μM). Following preincubation, cells were incubated with [^{3}H]choline for 1 h. Cells were collected, and the radiolabel in PC was determined. *C–E*, cytosols isolated from mice livers were assayed for choline kinase activity in the presence of various concentrations of different intermediates of purine metabolism. *F*, homogenates isolated from mice livers were preincubated with purine metabolites (500 μM) for 10 min, and then the active form of CT was assayed in the absence of PC/oleate. *G*, ATP levels in hepatocytes following AICAr incubation (105 min). *H*, the relationship between ATP concentration and PC biosynthesis rate in hepatocytes. Data are means ± S.D. from three separate hepatocyte preparations. The *asterisks* signify difference *versus* no AICAr treatment ($p < 0.05$). *HYP*, hypoxanthine; *INO*, inosine; *ASA*, adenylsuccinate.

choline kinase activity be inhibited independently of ethanolamine kinase? It is possible that the CK activity is more sensitive to changes in ATP concentrations than ethanolamine kinase. This postulation is supported by kinetic data indicating that the $K_{m(ATP)}$ for ethanolamine kinase is 0.5 mM compared with 3.7 mM for CK. This low K_m, compared with cellular ATP levels (3–5 mM) (43), makes ethanolamine kinase less sensitive to decreases in ATP concentrations in the range of 3 mM. In rodents, there exists at least one ethanolamine-specific kinase (45), which may further explain continued phosphoethanolamine production following AICAr incubation.

Physiological and Pathophysiological Implications—The maintenance of a high ratio of ATP to ADP is fundamental in all cells. In times of stress, such as exercise, hypoxia, and glucose deprivation, AMPK is activated to deal with changes in energy status (46, 47). In doing so, AMPK stimulates ATP-generating pathways, such as fatty acid oxidation, and reduces ATP-consuming processes, including cholesterol and TG biosynthesis (25–27). The plethora of AMPK-dependent actions is designed to normalize energy potential in the cell. We have shown that, unlike other anabolic processes, PC biosynthesis is not regulated by AMPK activation. However, the close dependence of choline kinase flux on ATP concentration may indicate a further "line of defense" against ATP depletion. PC is quantitatively the most important phospholipid in mammalian cells, and in liver, the equivalent of the entire PC pool is "turned over" in a 24-h period (48). The production of PC by the CDP-choline pathway requires both ATP and CTP; thus, PC biosynthesis has the potential to place a great demand on energy supply. It is unlikely that CTP is limiting following AICAr treatment, since the flux through CT, as measured by [^{3}H]glycerol incorporation into PC, was increased, not decreased. Therefore, it is possible that decreased CK flux could provide cells with a "short term" protection against ATP deprivation. Our study indicates that hepatocytes can survive for at least 2 h with the amount of PC produced from phosphocholine. However, if ATP levels are not normalized, prolonged incubations with AICAr are detrimental to hepatocytes. Recently, Li *et al.* (49) reported that a decrease in the cellular ratio of PC to PE causes steatohepatitis and liver failure. Our experiments agree with this finding, since 24-h AICAr incubations resulted in apoptotic hepatocytes with a decreased PC/PE ratio. It will be interesting to see if PC biosynthesis in other tissues, such as muscle and heart, is similarly regulated by cellular ATP supply.

Conclusion—It has been proposed that AICAr could be useful as an antidiabetic agent (50, 51). However, AICAr incubation in hepatocytes can result in inhibition of PC biosynthesis and ATP depletion. AICAr also behaves as a potent inhibitor of methylation reactions. In addition, we have shown that "long term" incubation with AICAr causes apoptosis, thus highlighting the potential side effects of such therapy. Nevertheless, it is clear that AICAr reduces hepatic PC biosynthesis, independently of AMPK activation, by inhibiting choline kinase flux. The discovery that choline kinase activity is sensitive to cellular ATP levels is an intriguing finding.

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