Regulation of phosphatidylcholine and phosphatidylethanolamine synthesis in rat hepatocytes by 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR)

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The present study was undertaken to study the role of AMP-activated kinase (AMPK) in the biosynthesis of two major membrane phospholipids, phosphatidylcholine (PC) and phosphatidylethanolamine (PE). Incubation of rat hepatocytes with 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR), an activator of AMPK, produced dose-dependent inhibition of the incorporation of [^{3}H]choline and [^{3}H]ethanolamine into PC and PE, respectively. Determination of the cellular uptake of choline and ethanolamine showed that the reduced synthesis of PC and PE did not result from impaired uptake of these two precursors. The decreased synthesis of PC was not mirrored by a reduction in the activities of the enzymes of the CDP-choline pathway. The diminution of PE biosynthesis, however, was paralleled by a depressed activity of CTP:phosphoethanolamine cytidylyltransferase (ET), the pace-setting enzyme of the CDP-ethanolamine pathway. AICAR treatment of hepatocytes stimulated the conversion of choline into betaine, indicating that reduced PC synthesis most probably resulted from a decrease in the availability of choline. In addition, AICAR induced a 50% reduction in the cellular level of diacylglycerols, which may further impair the synthesis of PC and PE. The results thus indicate that AICAR inhibits the biosynthesis of PC and PE and that the effect is exerted at different sites in the two pathways. Increased oxidation of choline to betaine is the main target of AICAR in the PC pathway, whereas inhibition of ET activity is the locus of AICAR action in the PE pathway.

Key words: AMP-activated protein kinase, choline oxidation, CTP:phosphoethanolamine cytidyltransferase, phospholipid synthesis.

INTRODUCTION

Phosphatidylcholine (PC) and phosphatidylethanolamine (PE) are quantitatively the most important phospholipids present in eukaryotic cell membranes [1,2]. In most mammalian tissues and cell types the major route for PC biosynthesis is the CDP-choline pathway, and that for PE biosynthesis is the CDP-ethanolamine pathway [1,2]. Another important route for the synthesis of PE is the decarboxylation of phosphatidylserine [2,3]. The contribution of each of these two pathways to overall PE synthesis in mammalian cells is not yet clear. Cells in culture do not require exogenous ethanolamine in the medium for growth, suggesting that under these conditions PE is formed mainly by phosphatidylserine decarboxylation [2,3]. On the other hand, studies *in vivo* with labelled substrates [4] and experiments with freshly isolated rat hepatocytes [5] have shown that the CDP-ethanolamine pathway may be an important route for hepatic PE synthesis *in vivo* when physiological concentrations of extracellular ethanolamine are present.

It is generally assumed that under most conditions CTP:phosphocholine cytidylyltransferase (CT; EC 2.7.7.15) and CTP:phosphoethanolamine cytidylyltransferase (ET; EC 2.7.7.14) are the major regulatory enzymes in the CDP-choline and CDP-ethanolamine pathways, respectively [1,3]. CT displays rather complex modes of control. Under different physiological conditions, changes in activity are governed by changes in compartmentation or in covalent phosphorylation [6,7]. Although the mechanism for membrane binding and subsequent activation of CT is unknown, soluble CT is highly phosphorylated at the C-terminus [8] and translocation of CT to membranes is accompanied by extensive dephosphorylation [9,10]. However, the precise role of reversible phosphorylation in the regulation of CT activity and its subcellular localization is yet not fully understood. In addition, little is known regarding the kinases and phosphatases that act on CT in the cell. Several proline-directed protein kinases have been shown to phosphorylate pure CT without altering its activity [11,12].

In contrast with the overwhelming amount of information present on the mechanisms involved in CT activation, not much is known about the mechanism(s) that regulate ET activity. Differential centrifugation studies [13,14] and enzyme-release measurements from digitonin-permeabilized hepatocytes [14] showed that ET, unlike CT, is a soluble protein. These data suggest that it is unlikely that ET activity would be controlled by a reversible translocation mechanism similar to that proposed for CT. However, Van Hellemond et al. [15] observed that ET displays a bimodal distribution between the cisternae of the rough endoplasmic reticulum (ER) and the cytosolic space. Additional support for association of ET with the ER or with CDP-ethanolamine:1,2-diacylglycerol ethanolaminephosphotransferase (EPT; EC 2.7.8.1), an integral ER protein [16], came from studies by Bladergroen et al. [17], showing channelling of intermediates in PE biosynthesis in rat fibroblasts. This suggests that the enzymes in the CDP-ethanolamine pathway are arranged in a 'metabolon'.

The last enzymes in the CDP pathways for PC and PE synthesis, CDP-choline:1,2-diacylglycerol cholinephosphotransferase (CPT; EC 2.7.8.2) and EPT, respectively, do not normally

Abbreviations used: PC, phosphatidylcholine; PE, phosphatidylethanolamine; AMPK, AMP-activated kinase; CT, CTP:phosphocholine cytidylyltransferase; CPT, CDP-choline:1,2-diacylglycerol cholinephosphotransferase; ET, CTP:phosphoethanolamine cytidylyltransferase; EPT, CDP-ethanolamine:1,2-diacylglycerol ethanolaminephosphotransferase; GPAT, *sn*-glycerol-3-phosphate acyltransferase; AICAR, 5-aminoimidazole-4-carboxamide ribonucleoside; ER, endoplasmic reticulum; DAG, diacylglycerol.

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determine the rate of phospholipid synthesis. However, in some cases, regulation at these steps, for instance via the supply of diacylglycerol (DAG) [3,6,18], can contribute to the overall control of PC and PE synthesis.

AMP-activated kinase (AMPK) belongs to a family of highly conserved serine kinases that are regulated by metabolic and nutritional stresses that increase cellular AMP concentrations. It has been suggested that the AMPK system evolved to protect the cell against ATP depletion by inhibiting biosynthetic pathways and stimulating energy-generating pathways. AMPK plays a major role in the regulation of lipid metabolism in mammals. Thus AMPK phosphorylates key regulatory enzymes of lipid metabolism, thereby changing their activity state. Examples of such enzymes are acetyl-CoA carboxylase, 3-hydroxy-3-methylglutaryl-CoA reductase and hormone-sensitive lipase [19]. It has been shown recently that treatment of hepatocytes with 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR), a compound that activates AMPK without changing the levels of cellular adenine nucleotides, inhibits the incorporation of radiolabelled oleate into phospholipids [20]. The authors showed that AMPK inhibits the mitochondrial isoform of *sn*-glycerol-3-phosphate acyltransferase (GPAT), the enzyme that catalyses the initial and committed step in glycerolipid biosynthesis. GPAT appears to represent a novel target of AMPK, whose inhibition decreases the *de novo* synthesis of triacylglycerol and phospholipids.

In the present study we focused on the effect of AICAR on the Kennedy pathways for PC and PE biosynthesis in freshly isolated hepatocytes. This model system offers the possibility of determining whether CT is a substrate for AMPK. Our results provide evidence that the synthesis of both PC and PE, via their respective CDP routes, are inhibited markedly by AICAR. The results show further that AICAR, possibly by activating AMPK, controls the synthesis of PE and PC synthesis at sites different from GPAT.

MATERIAL AND METHODS

Materials

Dulbecco's modified Eagle's medium and fetal bovine serum were from Gibco BRL, Paisley, Scotland, U.K. The radiochemicals [*methyl*-^{3}H]choline chloride (83 Ci · mmol^{-1}), [1-^{3}H]ethanolamine hydrochloride (17.9 Ci · mmol^{-1}), phospho[*methyl*-^{14}C]choline (56 mCi · mmol^{-1}), CDP [*methyl*-^{14}C]choline (54 mCi · mmol^{-1}), [2-^{14}C]ethanolamine hydrochloride (57 mCi · mmol^{-1}) and [γ-^{32}P]ATP (3000 Ci · mmol^{-1}) were from Amersham International, Little Chalfont, Bucks., U.K. Phospho[2-^{14}-C]ethanolamine was prepared enzymically from [2-^{14}C]ethanolamine with partially purified ethanolamine kinase as described previously [21]. *sn*-1,2-Diacylglycerol kinase was from Calbiochem, La Jolla, CA, U.S.A. 1,2-Dioleoylglycerol, L-α-phosphatidic acid, octyl-β-D-glucopyranoside, cardiolipin and choline were from Sigma, St. Louis, MO, U.S.A., and ethanolamine was from Baker, Deventer, The Netherlands. All other chemicals were of analytical grade.

Isolation and incubation of hepatocytes

Male Wistar rats (150–200 ***g***) were used throughout this study. Hepatocytes were isolated and incubated as described earlier [22]. Cells were suspended in an incubation medium consisting of Krebs/Ringer bicarbonate buffer (pH 7.4), 1.3 mM $CaCl_2$, 10 mM glucose and 1% (w/v) defatted and dialysed BSA. Triplicate incubations (final volume, 2.0 ml; cell concentration, 4–6 mg of protein/ml) were carried out in a metabolic shaker (85 oscillations/min) in 25 ml Erlenmeyer flasks under an atmosphere of O_2/CO_2 (19:1).

Uptake studies

For choline- and ethanolamine-uptake studies, cells were stopped as described in [23]. Briefly, 0.9 ml of incubation mixture was transferred to an Eppendorf tube containing 200 μl of ice-cold 2 M $HClO_4$ and approx. 400 μl of a mixture of bromododecane/bromodecane (3:1, v/v) on top of this layer. The tube was spun for 30 s at full speed in a modified Eppendorf 3200 centrifuge with a swing-out rotor. The protein precipitate below the bromododecane/bromodecane layer was resuspended with a glass rod to complete extraction of choline and ethanolamine into the perchloric acid, and re-centrifuged. Radiolabel taken up by the hepatocytes was quantified by counting an aliquot of the $HClO_4$ fraction for radioactivity.

Extraction and analysis by TLC of lipids and water-soluble metabolites

Lipids and water-soluble precursors were extracted from the cells by the method of Bligh and Dyer [24]. Phospholipids were separated by TLC on prefabricated silica G plates (Merck) in a solvent system of chloroform/methanol/water (65:35:4, by vol.). The aqueous phase was evaporated to dryness under nitrogen, and the water-soluble choline- or ethanolamine-containing metabolites were separated on silica HR plates in methanol/0.5% NaCl/ammonia (10:10:1, by vol.). In the above-described TLC system CDP-choline and betaine have the same R_F value. To separate these two compounds from the rest we used prefabricated silica G plates with methanol/0.5% NaCl/ammonia (6:3:2; by vol.) as a solvent system. Lipids and choline-containing compounds were visualized with iodine vapour, and ethanolamine-containing metabolites were visualized by spraying with 0.1% ninhydrin in ethanol. Spots were identified by comparison with known standards. The silica was scraped off the plate and the amount of radioactivity incorporated was determined by liquid-scintillation counting.

Subcellular fractionation

At the end of the incubation period, the cells were washed twice by centrifugation (100 ***g***) and resuspension with ice-cold homogenization buffer, containing 0.145 M NaCl, 10 mM Tris/HCl (pH 7.4), 1 mM EDTA and 10 mM NaF. The cells were homogenized and the homogenate was centrifuged subsequently at 105000 ***g*** for 60 min. The pellet (membrane fraction) was resuspended in a buffer containing 0.25 M sucrose, 10 mM Tris/HCl (pH 7.4), 1 mM EDTA and 10 mM NaF at a final concentration of 5 mg of protein/ml and used for measurement of the activities of CT, CPT and EPT. The 105000 ***g*** supernatant (soluble fraction) was used to determine the activities of choline and ethanolamine kinase, and CT and ET.

Enzyme assays

Choline and ethanolamine kinase activities were determined in the cytosolic fraction, as described in [25]. Labelled phosphocholine and phosphoethanolamine were separated from their respective radioactive substrates by paper chromatography (Whatman 3MM) with ethanol/propanol/ammonia (13:4:7, by vol.) as the developing solvent.

The assay for CT activity was performed in control or AICAR-treated hepatocytes essentially as described previously [26]. The CT activity in about 125 μg of membrane-bound or soluble protein was measured by the conversion of phospho[*methyl*-

^{14}C]choline to CDP[*methyl*-^{14}C]choline in 15 min at 37 °C. CT activity was assayed in the absence or presence of PC:oleic acid (1:1) vesicles. Radioactive CDP-choline and phosphocholine were separated by TLC on silica HR plates using methanol/0.5% NaCl/ammonia (10:10:1, by vol.) as the solvent system. CDP-choline and phosphocholine were applied as markers and visualized with iodine vapour. The silica spots containing CDP-choline were scraped off the plates and radioactivity was quantified by liquid-scintillation counting.

The activity of ET was measured in 80 μl of the soluble fraction (about 100 μg of protein), exactly as described by Tijburg et al. [21], except that phospho[2-^{14}C]ethanolamine and CDP[2-^{14}C]ethanolamine were separated on silica gel H TLC plates with 96% ethanol/0.5% NaCl/25% NH_4OH (10:10:1, by vol.) as the solvent.

CPT activity was assayed in a 50 μl membrane fraction (about 225 μg of protein) with endogenous DAG as the substrate, as described by Groener et al. [27]. The 100 μl assay mixture contained 20 mM Tris/HCl (pH 7.4), 10 mM $MgCl_2$, 4 mM dithiothreitol and 0.2 mM [*methyl*-^{14}C]CDP-choline (25000 d.p.m./nmol). The reactions were carried out at 37 °C for 10 min. The formation of radioactive PC was measured by the filter-disc method according to Goldfine [28].

Determination of ET activity in digitonin-permeabilized hepatocytes

ET activity was also determined in digitonin-permeabilized hepatocytes as the incorporation of radiolabelled phosphoethanolamine into CDP-ethanolamine using a new procedure. Hepatocytes were incubated (5 mg of protein/ml) in the presence or absence of 0.5 mM AICAR as described above. At different incubation times 100 μl aliquots were taken from the hepatocyte suspension, added to the ET/digitonin reaction mixture and vortexed gently for 3 s. The final assay mixture (300 μl) contained 20 mM Tris/HCl (pH 7.8), 10 mM $MgCl_2$, 1 mM EGTA, 5 mM dithiothreitol, 2 mM CTP, 1 mM phospho[2-^{14}C]ethanolamine (0.2 μCi/assay) and 50 μg of digitonin per mg of cell protein. After 6 min, reactions were stopped by the addition of 30 μl of 55% ice-cold trichloroacetic acid. The formed CDP-ethanolamine was separated from the radiolabelled substrate by TLC as described above. Preliminary experiments showed that, under the conditions used, the assay was linear with protein up to 750 μg and with time up to 8 min.

Determination of *sn*-1,2-DAG levels

sn-1,2-DAG was extracted from incubated hepatocytes and the amount was determined enzymically with diacylglycerol kinase exactly as described by Preiss et al. [29], except for the TLC procedure. [γ-^{32}P]Phosphatidic acid was separated on prefabricated silica G plates with chloroform/pyridine/formic acid (60:30:7, by vol.) as the developing solvent. Unlabelled phosphatidic acid was used as a standard. The silica-containing [γ-^{32}P]phosphatidic acid was scraped from the plates and the amount of radioactivity was measured by liquid-scintillation counting.

Other methods

Cell protein was measured as described by Lowry et al. [30], using BSA as a standard. Data are expressed as means ± S.D. All statistical analyses were done using unpaired Student's *t* tests.

RESULTS

AICAR inhibits the biosynthesis of PC and PE in isolated rat hepatocytes

Incubation of rat hepatocytes with AICAR resulted in the dose-dependent inhibition of [^{3}H]choline and [^{3}H]ethanolamine incorporation into PC and PE, respectively (Figure 1). Maximal inhibition was reached at a concentration of 0.5 mM. Treatment of hepatocytes with this concentration of AICAR inhibited the incorporation of labelled choline into PC by 78% and the incorporation of [^{3}H]ethanolamine into PE by 53% (Table 1). Furthermore, AICAR notably inhibited (91%) incorporation of labelled ethanolamine into PC via the three sequential methylations of PE, a reaction catalysed by PE-*N*-

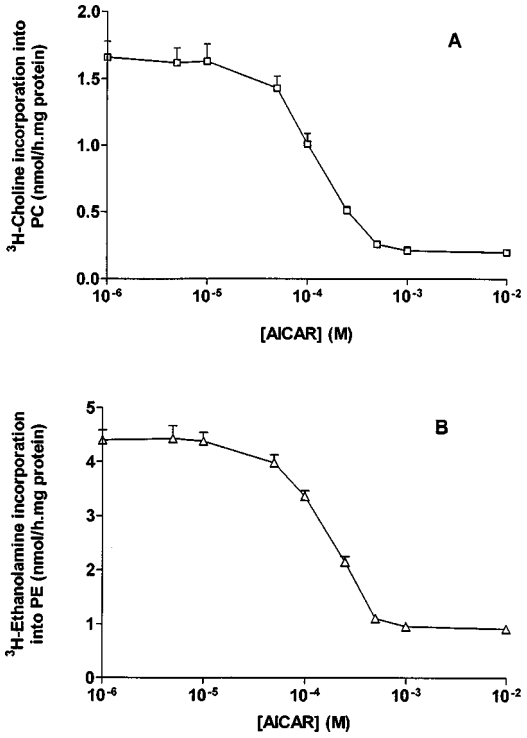

Figure 1 Effect of AICAR on the incorporation of labelled precursors into PC and PE

Isolated rat hepatocytes were pre-incubated in the absence or presence of various concentrations of AICAR. The incorporation of [^{3}H]choline into PC (**A**) and of [^{3}H]ethanolamine into PE (**B**) was determined after a 60 min pulse with the respective labels. Values are means ± S.D. from triplicate incubations of one representative experiment, which was repeated twice ($n = 3$).

Table 1 Effects of AICAR on the biosynthesis of PE and PC

Hepatocytes were incubated in the absence or presence of 0.5 mM AICAR for 30 min and pulsed subsequently for 1 h with the different labels. Results are expressed as means ± S.D. from three separate hepatocyte preparations. *Significantly different from control ($P < 0.01$).

	[^{3}H]Choline incorporation (nmol/mg of protein per h)	[^{3}H]Ethanolamine incorporation (nmol/mg of protein per h)	
	PC	PE	PC
Control	1.4 ± 0.18	5.49 ± 0.48	0.32 ± 0.04
AICAR	0.31 ± 0.05*	2.56 ± 0.17*	0.03 ± 0.01*

Table 2 Effects of AICAR on the activities of the enzymes of *de novo* PC synthesis

Hepatocytes were incubated for 90 min with or without 0.5 mM AICAR. Cells were homogenized and soluble and membrane fractions were prepared. The soluble fraction was assayed for choline kinase and CT activities, whereas CT and CPT activities were measured in the membrane fraction. Each value is the mean ± S.D. from three measurements. This experiment was repeated twice with similar results. The soluble fraction for CT activity was assayed in the presence of PC/oleate vesicles. Significantly different from controls: $^{*}P < 0.02$; $^{**}P < 0.01$.

	Specific activity (nmol/min per mg of protein)	
Enzyme	Control	AICAR
Choline kinase	5.74 ± 0.89	6.33 ± 0.43
CT (soluble fraction)	1.77 ± 0.21	1.25 ± 0.1*
CT (membrane fraction)	0.76 ± 0.04	1.46 ± 0.17**
CPT	0.33 ± 0.03	0.29 ± 0.02

methyltransferase (Table 1). Whether this effect was due to a decreased specific activity of PE or to an inhibition of the methyltransferase cannot be concluded from these experiments.

Determination of enzyme activities

In order to study the mechanism(s) responsible for the inhibition of PE and PC biosynthesis by AICAR we prepared subcellular fractions and determined the activities of the enzymes in the CDP-ethanolamine and CDP-choline routes, respectively. The activities of ethanolamine kinase (control, 1.14 ± 0.08 nmol/min per mg of protein; AICAR, 1.25 ± 0.11 nmol/min per mg of protein) and ET (control, 3.25 ± 0.44 nmol/min per mg of protein; AICAR, 3.13 ± 0.32 nmol/min per mg of protein) were not changed upon treatment of hepatocytes with AICAR. The activities of the first as well as the last enzymes in the CDP-

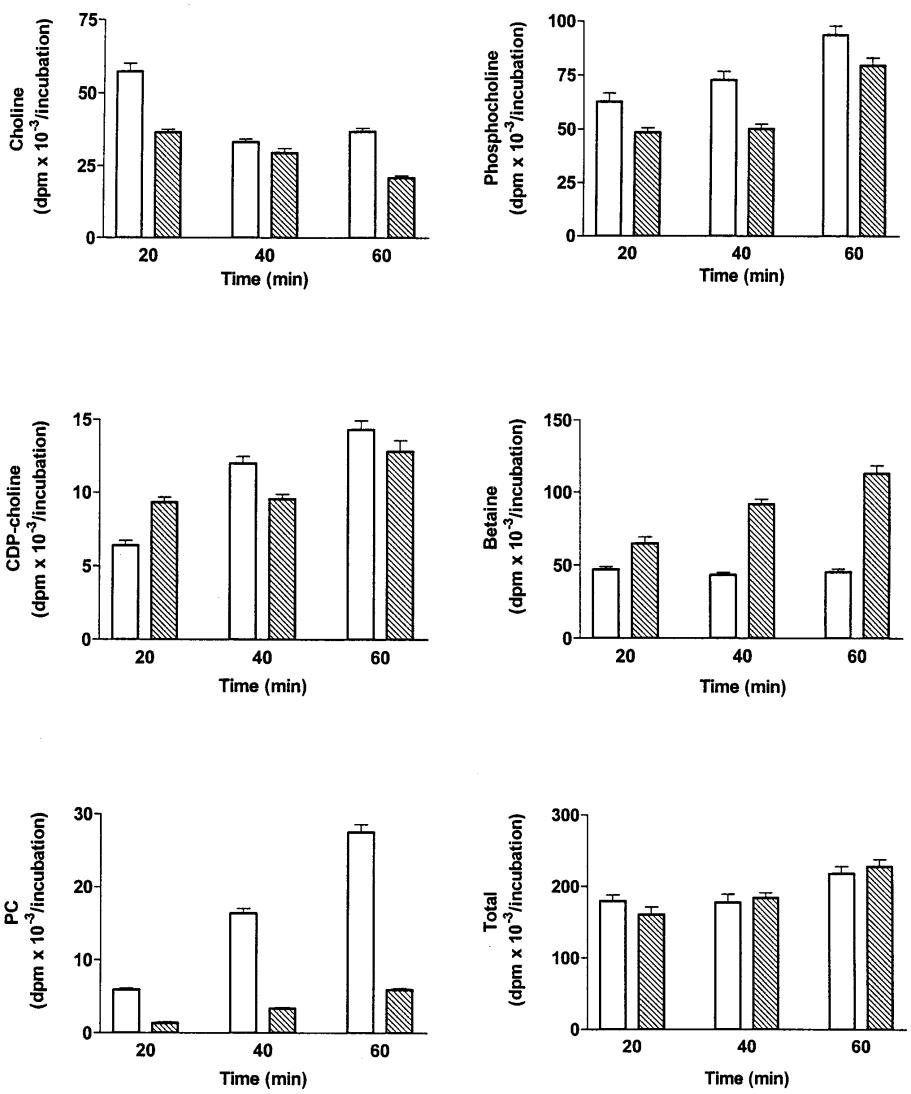

Figure 2 Effects of AICAR on the rate of incorporation of [^{3}H]choline into PC, choline intermediates and betaine

Hepatocytes were pre-incubated for 30 min in the absence (open bars) or presence (hatched bars) of 0.5 mM AICAR. The pulse (20, 40 or 60 min) was started with [^{3}H]choline. Data are expressed per incubation and are means ± S.D. from one representative experiment, which was repeated twice with similar results.

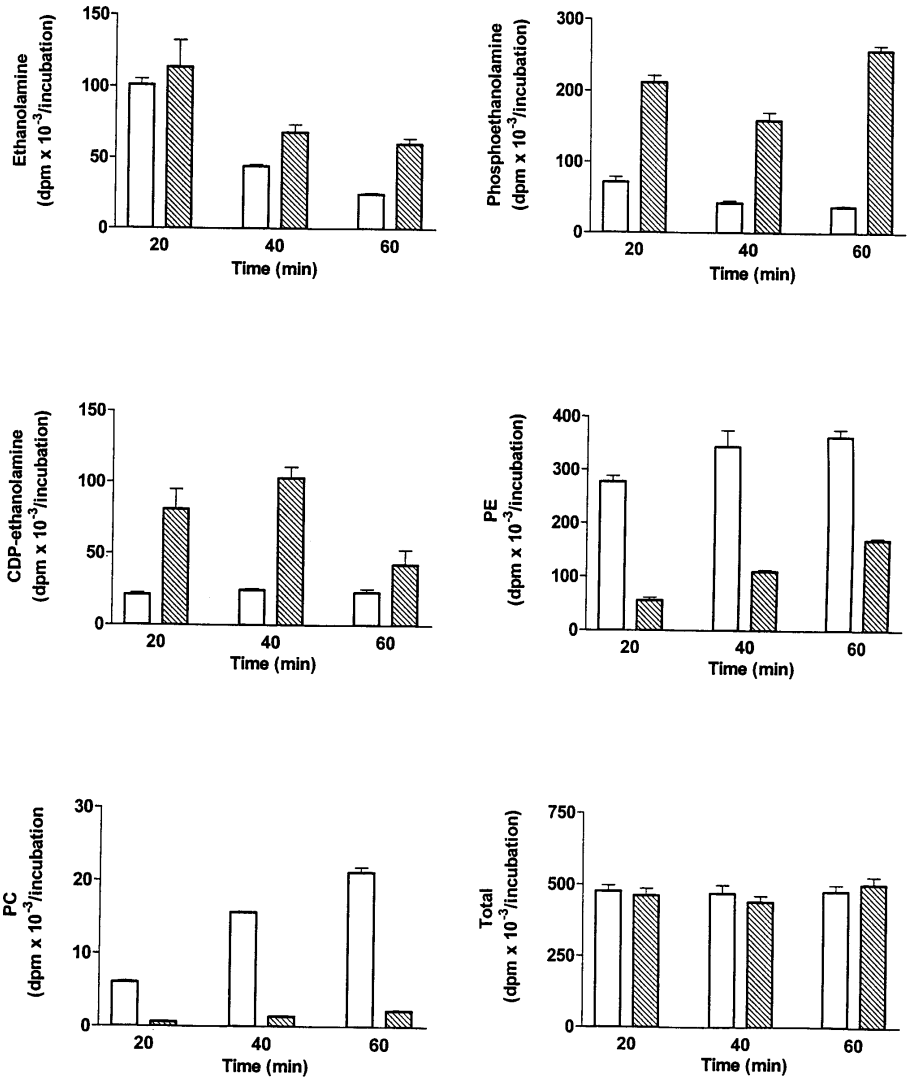

Figure 3 Effects of AICAR on the rate of incorporation of [^{3}H]ethanolamine into PE, PC and ethanolamine intermediates

Hepatocytes were pre-treated for 30 min in the presence (hatched bars) or absence (open bars) of 0.5 mM AICAR prior to a pulse with [^{3}H]ethanolamine for the times indicated. Data are expressed per incubation and are means ± S.D. from one representative experiment, which was repeated twice with similar results.

choline route, choline kinase and CPT, respectively, were not changed upon exposure of hepatocytes to AICAR, whereas CT activity was increased (Table 2). The latter was due to an AICAR-induced translocation of CT from the cytosol to the membranes, corresponding to activation of the enzyme [1,6].

Uptake studies

It has been shown previously [31,32] that some compounds which alter the incorporation of labelled choline into PC exert their effect at the level of choline uptake. The observation that AICAR-induced inhibition of PC and PE biosynthesis was not mirrored by an inhibitory effect on the activities of the three enzymes in the respective CDP routes prompted us to determine whether a change in the rate of choline and/or ethanolamine uptake could explain the observed reduction in phospholipid synthesis. Choline and ethanolamine uptake were studied for different time periods up to 8 min. The data demonstrated clearly that up to 8 min the amount of intracellular choline (including choline metabolites) was not significantly different between control and AICAR-treated cells. Similar results were obtained when the uptake of ethanolamine was studied (results not shown).

To gain insight into the distribution of label between the different water-soluble intermediates in both CDP routes, aliquots of the 8 min time point were analysed. Unexpectedly, a large amount of label was associated with the CDP-choline fraction, which increased significantly upon AICAR treatment (results not shown). At this point we hypothesized that the high amount of label in the CDP-choline pool was due to the solvent system used, in which CDP-choline runs at the same R_F value as betaine. Betaine is synthesized in the liver by the oxidation of choline, a reaction catalysed by choline oxidase [33]. In the next set of experiments, in which the water-soluble intermediates in the CDP routes were studied, a solvent system was used in which CDP-choline and betaine were separated.

Table 3 Effect of AICAR on the activity of ET as measured in the digitonin-permeabilized cell assay

Cells were incubated with or without 0.5 mM AICAR for different times. After 30, 60 or 90 min an aliquot of the cell suspension was assayed for ET activity using the digitonin-permeabilized cell assay. Values are expressed as means ± S.D. from three different cell populations. Significantly different from control incubations: $^{*}P < 0.02$; $^{**}P < 0.01$.

	Specific activity (nmol/min per mg of protein)		
Incubation time (min)	Control	AICAR	Inhibition (%)
30	3.03 ± 0.09	2.59 ± 0.15*	15
60	3.08 ± 0.04	2.22 ± 0.12**	28
90	3.02 ± 0.29	2.02 ± 0.11**	33

Table 4 Effect of AICAR on the amount of DAGs

Hepatocytes were incubated for 30 or 90 min in the absence or presence of 0.5 mM AICAR. Values are means ± S.D. from triplicate incubations of a representative experiment, which was repeated twice ($n = 3$) with similar results. *Significantly different from control incubations ($P < 0.001$).

	Amount of diacylglycerol (nmol/mg of protein)	
Incubation time (min)	Control	AICAR
30	6.61 ± 0.24	2.81 ± 0.32*
90	7.58 ± 0.31	3.64 ± 0.32*

Water-soluble intermediates

Hepatocytes were pulsed with [^{3}H]choline or [^{3}H]ethanolamine for various times (up to 60 min) in the absence or presence of AICAR, and label in the different water-soluble intermediates and phospholipids was determined. It is clear from Figure 2 that in hepatocytes a large amount of betaine is synthesized from choline, supporting our hypothesis that the unexpectedly high amount of label associated with the CDP-choline fraction was due to betaine. Treatment of the cells with AICAR had no effect on the total amount of label in the CDP-choline pool, whereas the amount of betaine increased significantly upon exposure to AICAR (Figure 2).

The amount of label in both the choline and phosphocholine fractions was reduced. The decrease in the choline fraction is probably due to the increased oxidation of choline to betaine. The elevated conversion of choline into betaine may also account for the decreased amount of label in the phosphocholine pool. However, an increase in CT activity, as was seen in AICAR-treated hepatocytes (see Table 2), may also have been responsible. Furthermore, it is clear from Figure 2 that the effect of AICAR on PC synthesis was rapid and that the total amount of labelled choline taken up by the hepatocytes did not change in the time period studied.

Rapid inhibition of PE synthesis was seen when hepatocytes were treated with AICAR, whereas the total amount of ethanolamine taken up by the cells did not change (Figure 3). The amount of radiolabel in all three intermediates in the CDP-ethanolamine pathway was elevated by AICAR. The increased amount of label in the phosphoethanolamine pool suggests an inhibition of ET and/or EPT activity.

Effect of AICAR on the activity of ET, as measured in a digitonin-permeabilized cell assay

ET activity in hepatocytes did not change upon AICAR treatment when activity was assayed in the soluble fraction. It has been shown previously [15] by electron-microscopy studies that ET associates with the rough ER. On the other hand, classical cell-fractionation procedures showed that all ET activity is always in the soluble fraction. The combination of these two observations suggests a weak association of ET with the rough ER, which is probably disturbed upon homogenization of the cells. Therefore, ET activity was measured using a digitonin procedure that had been shown to circumvent post-homogenizing modification [34]. Such a method appeared previously useful for acetyl-CoA carboxylase, another so-called cytosolic enzyme [34]. The latter enzyme is most probably associated weakly with the cytoskeleton [35].

It is clear from Table 3 that ET activity, when measured using the digitonin method, is inhibited by 30–40 % upon AICAR treatment. This result is in agreement with the accumulation of label in the phosphoethanolamine pool and the overall inhibition of PE biosynthesis.

Effect of AICAR on the amount of intracellular DAGs in hepatocytes

It has been shown that the amount of DAG is one of the factors determining the rate of PC as well as PE biosynthesis [1,3,18]. Muoio et al. [20] showed that AICAR inhibits the incorporation of labelled oleate and glycerol into DAG at the level of mitochondrial GPAT. However, whether the inhibition of GPAT resulted in a reduced amount of DAG was not determined. Table 4 shows that incubation of hepatocytes with AICAR resulted in a significant decrease (55 %) in the amount of intracellular DAG.

DISCUSSION

AMPK has been described as a cellular 'fuel gauge' that is activated when metabolic stresses deplete ATP. AMPK responds to the increased cellular AMP/ATP ratio by inhibiting biosynthetic pathways that use energy, and by stimulating pathways that produce energy [36]. AMPK has been implicated in the regulation of a number of pathways in lipid metabolism. However, its role in the regulation of phospholipid metabolism, especially in the biosynthesis of two major membrane phospholipids, PC and PE, has not been studied to date.

In hepatocytes, AICAR, at the concentration used in our experiments (0.5 mM), stimulated AMPK up to 12-fold without changing the cellular concentration of ATP, AMP or ADP [37]. Muoio et al. [20] have proposed a model for the reciprocal regulation of fatty acid oxidation and triacylglycerol biosynthesis by AMPK in hepatocytes. They showed, using labelled oleate, that in isolated hepatocytes triacylglycerol, DAG and phospholipid synthesis were inhibited by AICAR. Inhibition of the *de novo* synthesis of these glycerolipids was also seen using [^{3}H]-glycerol as a label, suggesting regulation at an early step in glycerolipid synthesis. The activity of GPAT, which catalyses the first and committed step in glycerolipid synthesis, was shown to be decreased upon AICAR treatment. Addition of AICAR to rat hepatocytes inhibited mitochondrial but not microsomal GPAT activity. Mitochondrial GPAT is developmentally, nutritionally and hormonally regulated [38,39], but its precise role remains elusive because mitochondria lack the enzymic machinery necessary to synthesize glycerolipids beyond lysophosphatidic acid [40,41]. We studied the effect of AICAR on the CDP-choline and CDP-ethanolamine pathways, because (i) the reported effect of AICAR on mitochondrial GPAT activity was rather small [20], (ii) the authors did not discriminate between the different

major phospholipid classes and (iii) AICAR may potentially regulate the synthesis of PC and PE by a phosphorylation/dephosphorylation mechanism of the rate-regulatory enzymes in the pathways to synthesize these two major membrane phospholipids.

It could be argued that the effects of AICAR on phospholipid synthesis in hepatocytes are mediated through activation of the adenosine receptor. However, this is not very likely, since incubation of hepatocytes with (i) adenosine (up to 50 μM) had no effect on PC or PE synthesis, (ii) AICAR in the presence of adenosine deaminase did not attenuate the effect of AICAR on phospholipid synthesis and (iii) CGS-15943, a highly potent non-selective antagonist of the adenosine receptor, did not affect phospholipid synthesis and did not modulate the effect of AICAR on both PC and PE synthesis (results not shown).

Our data support the model of Muoio et al. [20] for the reciprocal regulation of glycerolipid synthesis and fatty acid oxidation. In addition, the results of the present study show that more factors are responsible for the inhibition of PC and PE biosynthesis in hepatocytes treated with AICAR besides the reported decrease in activity of mitochondrial GPAT. These are (i) increased oxidation of choline to betaine, (ii) decreased activity of ET and (iii) decreased availability of DAG.

It has been shown before that, under certain conditions, the rate of PC and PE biosynthesis via their respective CDP routes is inhibited by the supply of DAG [1,3,18]. However, the activity of CPT measured in microsomes with endogenous DAG as substrate was not changed upon AICAR treatment of hepatocytes. This suggests that the amount of DAG present in the microsomal fraction is not rate limiting at the concentration present in microsomes isolated from AICAR-treated hepatocytes. Whether the same is the case for the situation *in vivo* is not known.

An unexpected observation was that treatment of hepatocytes with AICAR resulted in activation of CT, whereas the rate of PC synthesis was markedly reduced. We have no ready explanation for this, yet a similar phenomenon has been reported in choline-deficient rats [42,43]. The authors showed that in choline-deficient livers a decreased rate of PC biosynthesis was accompanied by translocation of CT to the ER. The mechanism for the reversible translocation of CT in this model system was a reduced amount of PC in the ER upon choline deprivation [43]. It does not appear very likely that a similar mechanism is responsible for the activation of CT in AICAR-treated hepatocytes, because short-term inhibition of PC synthesis (60 min) is probably not accompanied by a decrease in PC mass. However, in view of the channelling hypothesis [17,44], we speculate that the increased oxidation of choline results in the decreased availability of phosphocholine (see Figure 2), which is 'sensed' by CT, and, as a kind of rescue mechanism, triggers its activation.

Measurement of the activity of ET in the cytosol after subcellular fractionation of the hepatocytes showed no change following AICAR treatment of intact cells. However, when ET activity was determined using the digitonin procedure, an inhibition of its activity (35%) was found in AICAR-treated hepatocytes. An explanation for this discrepancy could be the localization of ET in hepatocytes. In contrast with the general belief, Van Hellemond et al. showed that ET is enriched in areas of the cell that are rich in rough ER [15]. Although no evidence is available that the amount of ET associated with the ER changes upon inhibition or stimulation of PE biosynthesis, or about whether the ER-associated ET is more active than soluble ET, it is interesting to hypothesize that, in analogy with CT, this may be the case. In view of the very weak membrane association of ET [15] it is plausible to assume that the effect is lost upon subcellular fractionation of the hepatocytes. On the other hand, when ET activity was measured in an *in vivo*-like situation using the digitonin assay an effect of AICAR was detectable. An interesting observation is that although the CDP-choline and CDP-ethanolamine routes are quite similar, AICAR exerts its inhibiting effect at different levels in the two pathways.

What is the physiological relevance of the increased oxidation of choline by AICAR in hepatocytes? It has been proposed that betaine serves as an important methylating agent when normal methylating pathways are impaired by ethanol ingestion, drugs or nutritional imbalances [45]. AICAR is a compound that mimics metabolic stress (high AMP/ATP ratio). It is possible that in such a situation AMPK plays a role in providing enough methylating agents.

In conclusion, the observations presented here suggest that AICAR-induced reduction of PC and PE biosynthesis is regulated by different mechanisms. Increased oxidation of choline to betaine is the main target of AICAR in the PC-biosynthetic pathway, whereas inhibition of ET is a major locus of AICAR action in PE synthesis. We hypothesize that ET activity is regulated by a phosphorylation mechanism and that the target of AICAR's action on PE biosynthesis in this system is actually AMPK. However, the present study does not provide direct evidence for this hypothesis. Further work will be directed to investigate this possibility.

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