AMP-activated kinase reciprocally regulates triacylglycerol synthesis and fatty acid oxidation in liver and muscle: evidence that *sn*-glycerol-3-phosphate acyltransferase is a novel target

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AMP-activated kinase (AMPK) is activated in response to metabolic stresses that deplete cellular ATP, and in both liver and skeletal muscle, activated AMPK stimulates fatty acid oxidation. To determine whether AMPK might reciprocally regulate glycerolipid synthesis, we studied liver and skeletal-muscle lipid metabolism in the presence of 5-amino-4-imidazolecarboxamide (AICA) riboside, a cell-permeable compound whose phosphorylated metabolite activates AMPK. Adding AICA riboside to cultured rat hepatocytes for 3 h decreased [^{14}C]oleate and [^{3}H]glycerol incorporation into triacylglycerol (TAG) by 50 % and 38 % respectively, and decreased oleate labelling of diacylglycerol by 60 %. In isolated mouse soleus, a highly oxidative muscle, incubation with AICA riboside for 90 min decreased [^{14}C]oleate incorporation into TAG by 37 % and increased $^{14}CO_2$ production by 48 %. When insulin was present, [^{14}C]oleate oxidation was 49 % lower and [^{14}C]oleate incorporation into TAG was 62 % higher than under basal conditions. AICA riboside blocked insulin's antioxidative and lipogenic effects, increasing fatty acid oxidation by 78 % and decreasing labelled TAG 43 %. Similar results on fatty acid oxidation and acylglycerol synthesis were observed in C_2C_{12} myoblasts, and in differentiated C_2C_{12} myotubes, AICA riboside also inhibited the hydrolysis of intracellular TAG. These data suggest that AICA riboside might inhibit *sn*-glycerol-3-phosphate acyltransferase (GPAT), which catalyses the committed step in the pathway of glycerolipid biosynthesis. Incubating rat hepatocytes with AICA riboside for both 15 and 30 min decreased mitochondrial GPAT activity 22–34 % without affecting microsomal GPAT, diacylglycerol acyltransferase or acyl-CoA synthetase activities. Finally, purified recombinant AMPKα1 and AMPKα2 inhibited hepatic mitochondrial GPAT in a time- and ATP-dependent manner. These data show that AMPK reciprocally regulates acyl-CoA channelling towards β-oxidation and away from glycerolipid biosynthesis, and provide strong evidence that AMPK phosphorylates and inhibits mitochondrial GPAT.

Key words: lipid partitioning, hepatocytes, myocytes.

INTRODUCTION

After entering cells, long-chain fatty acid is converted into acyl-CoA, which may then be either β-oxidized or esterified into complex lipids, including triacylglycerol (TAG). Adjustments in acyl-CoA partitioning between oxidative and biosynthetic pathways are critical responses during metabolic stresses that deplete energy, such as exercise and starvation. In response to energy depletion, fatty acid oxidation is increased, and one may predict that fatty acid storage in the form of TAG would be reciprocally decreased. AMP-activated protein kinase (AMPK) is a probable mediator of acyl-CoA partitioning into glycerolipid synthesis versus β-oxidation.

AMPK belongs to a family of highly conserved serine kinases that are regulated by metabolic and nutritional stresses that increase cellular AMP concentrations [1]. AMP allosterically activates AMPK and potentiates AMPK activity by stimulating an upstream kinase, AMPK kinase, which phosphorylates and activates AMPK [2]. In addition, the binding of AMP to AMPK renders the kinase a better substrate for its activator, AMPK kinase, and a worse substrate for protein phosphatases that might inactivate it [3]. *In vitro*, high concentrations of ATP antagonize the stimulation of AMPK by AMP, and therefore the cellular ratio of AMP:ATP appears to be the primary regulator of AMPK activity.

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 [1]. In keeping with this model, AMPK phosphorylates and inactivates liver acetyl-CoA carboxylase (ACC), 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase, glycogen synthase [4,5] and creatine kinase [6], enzymes that control the synthesis of fatty acids, cholesterol, glycogen and phosphocreatine respectively. The product of ACC, malonyl-CoA, is both a precursor for the biosynthesis of fatty acids and a potent inhibitor of β-oxidation because it inhibits carnitine palmitoyltransferase I (CPT1) and, thereby, the transport of long-chain fatty acids into the mitochondrial matrix [7]. Thus when ACC is inactivated, cellular concentrations of malonyl-CoA decrease, the inhibition of CPT1 is relieved, and more fatty acid is oxidized. AMPK has

Abbreviations used: ACC, acetyl-CoA carboxylase; ACS, acyl-CoA synthetase; AICA, 5-amino-4-imidazolecarboxamide; AMPK, AMP-activated kinase; ASM, acid-soluble metabolites; CPT1, carnitine palmitoyltransferase 1; DAG, diacylglycerol; DGAT, diacylglycerol acyltransferase; DMEMH, Dulbecco's modified high-glucose Eagle's medium; FBS, fetal bovine serum; GPAT, *sn*-glycerol-3-phosphate acyltransferase; HMG-CoA, 3-hydroxy-3-methylglutaryl-CoA; MEM, minimal essential medium; NEM, *N*-ethylmaleimide; TAG, triacylglycerol.

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been shown to increase fatty acid oxidation in liver, heart and skeletal muscle, the primary tissues that oxidize fatty acids [8,9].

We hypothesized that AMPK would also inactivate one or more of the enzymes of glycerolipid biosynthesis, thereby inhibiting TAG synthesis and promoting the partitioning of fatty acyl-CoA towards β-oxidation. To test our hypothesis, we incubated liver and muscle with 5-aminoimidazole-4-carboxamide (AICA) riboside, a cell-permeable compound that is metabolized to its monophosphorylated derivative, ZMP (an AMP analogue), which mimics the multiple stimulatory effects of AMP as an activator of AMPK [10]. We provide direct evidence 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 TAG and phospholipids. By inactivating both ACC and GPAT, AMPK would regulate the partitioning of fatty acid between oxidative and biosynthetic pathways, in both a co-ordinated and reciprocal manner.

EXPERIMENTAL

Materials

[^{3}H]Glycerol and [^{14}C]oleate were obtained from Amersham Life Sciences Co. (Arlington, IL, U.S.A.). AICA riboside, BSA (essentially fatty-acid-free), glucose, carnitine, sodium oleate and horse serum were from Sigma (St. Louis, MO, U.S.A.). Silica-gel plates and filter paper were from Whatman (Maidstone, Kent, U.K.). Tissue culture supplies and fetal bovine serum (FBS) were from Life Technologies, Inc. (Gaithersburg, MD, U.S.A.).

Animals

All animal protocols were approved by the University of North Carolina at Chapel Hill Institutional Animal Care and Use Committee. Hepatocytes were isolated from livers of male Sprague–Dawley rats (200–250 g). Soleus muscles were isolated from female C57BL/6J mice (Jackson Laboratory, Bar Harbor, ME, U.S.A.). Animals were maintained on a 12-h/12-h light/dark cycle with free access to Purina mouse chow, which was removed from the animals' cages at 09:00h during the experiments.

Hepatocyte isolation and incubations

Hepatocytes were isolated by collagenase perfusion [11]. Cell viability, determined by Trypan Blue exclusion, always exceeded 95%. Hepatocytes were seeded at a density of 200000 cells/well into 24-well dishes (Becton Dickinson and Co., Lincoln Park, NJ, U.S.A.) that had been pretreated with 0.01% (w/v) rat-tail collagen in 0.1 M acetic acid, and cultured at 37 °C in a humidified atmosphere of 5% CO_2 in 2.0 ml of minimal essential medium (MEM) containing 10% (v/v) heat-inactivated FBS, 50 units/ml penicillin and 50 μg/ml streptomycin. After cells had attached for 3–4 h, media was replaced with serum-free MEM plus 10 nM dexamethasone and 0.1 mM non-essential amino acids, and hepatocytes were cultured overnight. Experiments were performed the following morning in media with serum-free MEM containing 10 nM dexamethasone, 10 mM Hepes, 1.0 mM carnitine, 0.5% (w/v) fatty-acid-free BSA and 0.2 mM sodium oleate, with either [^{14}C]oleate (0.8 μCi/mM) or 0.5 mmol/ml [^{3}H]glycerol (1.0 Ci/mM) in the presence or absence of 0.5 mM AICA riboside. After 1–3 h, the media were transferred to new dishes and assayed for labelled oxidation products [CO_2 and acid-soluble metabolites (ASM)]. Hepatocytes were washed twice with 1% (w/v) BSA in PBS at 37 °C, scraped in two additions of 0.5 ml of ice-cold methanol and 0.5 ml of water, and total cell lipids were extracted [12]. In pulse–chase experiments, cells were labelled for 12 h in media containing 0.2 mM [^{14}C]oleate (as above), washed twice, and then incubated for 3 h in media containing 0.2 mM unlabelled sodium oleate in the presence or absence of 0.5 mM AICA riboside. Media and cells were collected and analysed as described above.

In cell-suspension experiments, hepatocytes (750000 cells/ml) were incubated in Krebs–Henseleit Buffer bicarbonate buffer/5.0 mM glucose/1.0 mM carnitine/1% (w/v) BSA, with 0.2 mM [^{14}C]oleate (0.8 μCi/mM). Incubations for 1–2 h in the absence or presence of 0.5 mM AICA riboside were performed in a total volume of 1.5 ml of media in 24-well dishes that were agitated in a gyrating bath at 37 °C under a constant atmosphere of O_2/CO_2 (19:1). To terminate the experiments, cells were transferred to 1.5 ml of ice-cold microcentrifuge tubes and centrifuged for 3 min at 3000 rev./min (1600 ***g***) at 5 °C. The cell pellet was washed twice with 1% (w/v) BSA in PBS and added to 0.5 ml of ice-cold methanol for lipid extraction [12]. In separate dishes, incubations were terminated by adding 100 ml of 70% (v/v) perchloric acid to the incubation media, which was subsequently assayed for $^{14}CO_2$ and ASM.

Myocyte cell cultures

Monolayers of mouse C_2C_{12} myoblasts (A.T.C.C. CRL-1772) from the American Type Culture Collection (Rockville, MD, U.S.A.) were grown in Dulbecco's modified high-glucose Eagle's medium (DMEMH; 90%)/10% (v/v) FBS/4.0 mM glutamine/50 μg/ml gentamycin, in a humidified incubator at 37 °C, 5% CO_2. Cells were grown in 100-mm dishes, subcultured at 60–80% confluence, and split at a ratio of 1:10 using trypsin [0.25% (w/v) in MEM with 1.0 mM EDTA]. Cells grown to 60% confluence were subcultured at a ratio of 1:15 into 6-well dishes that had been coated with 0.01% (w/v) collagen. When cells were 80% confluent, myoblasts were induced to differentiate into myotubes by changing to low-serum differentiation medium [98% DMEMH/2% (v/v) horse serum/4.0 mM glutamine/25 mM Hepes/50 μg/ml gentamycin]. Differentiation medium was changed daily. By day 5, cells were fully confluent and had differentiated into multinucleated, contracting myotubes.

Myocytes maintained in differentiation medium for 0 (myoblasts) or 5–6 days (myotubes) were preincubated for 1 h in serum-free MEM/12.0 mM glucose/4.0 mM glutamine/25 mM Hepes/1% (w/v) BSA/0.25 mM oleate/50 μg/ml gentamycin. Preincubation medium was replaced with 1.0 ml of fresh media containing [^{14}C]oleate (1.0 μCi/mM), and cells were incubated for 2 h at 37 °C in the absence or presence of 1.0 mM AICA riboside and 10^{-7} M insulin. Incubations were terminated by removing the media, which were subsequently assayed for ^{14}C-labelled oxidation products (see below). The cells were washed twice with 1% (w/v) BSA in PBS at 37 °C, scraped in two additions of 1.0 ml of ice-cold methanol and 0.5 ml of water, and lipids were extracted [12]. For pulse–chase experiments, cells were pre-labelled for 3 h in pulse media containing 0.25 mM [^{14}C]oleate, as described above. Cells were washed twice with 1% (w/v) BSA in PBS, and then incubated for 3 h in chase media containing 0.25 mM unlabelled sodium oleate in the presence or absence of 1.0 mM AICA riboside. Media and cells were collected and analysed as described above.

Muscle isolation and incubations

Mouse soleus muscles were removed between 09:00h and 11:00h under anaesthesia (100 mg/kg ketamine and 10 mg/kg xylazine),

cleaned free of adipose and connective tissue, and immediately transferred to a 24-well tissue-culture dish in a shaking water bath at 29 °C. Each well contained 1.0 ml of a modified Krebs–Ringer bicarbonate buffer (KRB) [low-calcium Kreb's Henseleit bicarbonate buffer/1.0 % (w/v) dialysed BSA/5.0 mM glucose], and was gassed continuously with O_2/CO_2 (19:1). After all muscles were isolated, they were incubated for an additional 15 min at 37 °C, before being transferred to fresh KRB medium containing oleate (1.0–1.5 mM), 1.0 mM carnitine and 1 % (w/v) BSA (KRB + fatty acid medium) and, where indicated, 10^{-7} M insulin. Muscles were incubated for 20 min in KRB + fatty acid medium, and then transferred to fresh KRB + fatty acid medium containing [^{14}C]oleate (1.0 μCi/ml) and, where indicated, 10^{-7} M insulin. High oleate concentrations were used in order to inhibit lipolysis of intramuscular TAG, and thereby minimize label dilution. AICA riboside (0.5–2.0 mM) was added to half the muscles, and the contralateral muscle from each mouse was used as a basal (non-treated) control. Following a 90 min incubation, muscles were washed twice in ice-cold KRB for 10 min, weighed and immediately frozen at −80 °C until extracted for lipids. Incubation medium was then assayed for oxidation products as described below.

Determination of fatty acid partitioning

To assay oxidation products, semi-dry filter paper (Whatman #3) saturated with 2 M NaOH was placed over 24-well incubation dishes and tightly covered with a foam pad and the plate cover. $^{14}CO_2$ produced by muscles or cells was driven from the media to the filter-paper trap by adding 100 μl of 70 % (v/v) perchloric acid to each well. After 60 min in a shaking bath at 37 °C, the filter paper discs corresponding to each well were dried, cut and washed in 2.0 ml of distilled water. $^{14}CO_2$ was quantified in 1.0-ml aliquots of the water wash. ASM, a measure of ketone bodies in the liver and of tricarboxylic-acid-cycle intermediates and acetyl esters in muscle [13], were assayed in supernatants of the acid precipitate. In hepatocytes, ASM routinely accounted for over 90 % of total oxidation products. Minimal increases in ^{14}C over background levels occurred in media in which muscles or myocytes had been incubated, indicating that insignificant amounts (< 15 % of the counts recovered as CO_2) of ASM had been released into the medium.

Lipids were extracted from muscles or cells and TAG, diacylglycerol (DAG), phospholipids and fatty acids were separated by thin-layer chromatography on scored silica gel G plates in hexane:diethyl ether:acetic acid (80:20:1, v/v) [14]. Radioactivity incorporated into different lipid species was determined by comparison with standards using a Bioscan 200 system.

Enzyme extraction and assays

To measure enzyme activities, hepatocytes were diluted to a density of 10^7 cells/ml in media identical with that of the hepatocyte suspensions, including 0.2 mM unlabelled oleate. A fraction of the cell suspension (2 ml) was incubated for 0–90 min in 6-well dishes that were agitated in a gyrating bath at 37 °C under O_2/CO_2 (19:1). Incubations were terminated by adding the cells to 1.0 ml of 3 × homogenization buffer (0.75 mM sucrose/30 mM Tris base/3.0 mM EDTA/3.0 mM dithiothreitol/150 mM sodium fluoride/12 mM sodium pyrophosphate) at pH 7.4, and then quickly frozen in a 95 % ethanol/dry-ice slurry. As cells thawed they were homogenized in a motor-driven Teflon–glass homogenizer using 10 up-and-down strokes. Homogenates were centrifuged at 100000 ***g*** for 60 min. The total particulate pellet was re-homogenized using 5 up-and-down strokes in homogenization buffer, and aliquots were stored at −80 °C until enzyme assays were performed. Mitochondrial fractions from rat liver were obtained by differential centrifugation [15].

Total GPAT activity was measured with 300 μM [^{3}H]glycerol 3-phosphate and 25 μM palmitoyl-CoA [16]. The mitochondrial GPAT activity was measured after incubating membranes for 15 min on ice with *N*-ethylmaleimide (NEM). NEM inhibits microsomal GPAT, but not mitochondrial GPAT [17]. NEM-resistant GPAT activity (mitochondrial) was subtracted from the total activity to determine microsomal GPAT activity. Diacylglycerol acyltransferase (DGAT) activity was measured with 30 μM [^{3}H]palmitoyl-CoA and 200 μM *sn*-1,2-dioleoylglycerol [18]. Acyl-CoA synthetase (ACS) was measured with 50 μM [^{3}H]palmitate/10 mM ATP/0.2 mM CoA [19].

AMPK inhibition

AMPK heterotrimers, containing either α-1 or α-2 catalytic subunits and with β-1 and γ-1 non-catalytic subunits, were isolated after cDNA expression in COS-7 cells [20]. Rat liver mitochondria (150 μg of protein) were incubated with AMPK1 or AMPK2 [20] in 0.2 mM AMP/5.0 mM $MgCl_2$/120 mM NaCl/80 mM Hepes, pH 7.0, for 10–30 min at 30 °C in the presence or absence of 1.3 mM ATP, before being assayed for GPAT activity.

Other methods

Cellular DNA content was measured fluorimetrically [21]. Protein concetration was measured using BSA as the standard [22]. [^{3}H]Glycerol 3-phosphate [23] and [^{3}H]palmitoyl-CoA [24] were synthesized enzymically.

Statistics

Data from muscle incubations are presented as means ± S.E.M. Differences between basal and AICA riboside-treated contralateral muscles were analysed by paired Student's *t* test at the $P < 0.05$ level. All other data are presented as means ± S.D. and were analysed by either Student's *t* test or by two-way (treatment × time) analysis of variance. Statistically significant differences between hormone treatments were detected by Newman–Keuls' post-hoc test.

RESULTS

AMPK regulates glycerolipid metabolism in hepatocytes

When primary rat hepatocytes in monolayer culture were incubated with either [^{14}C]oleate or [^{3}H]glycerol, oleate incorporation into complex lipids and oxidation products (ASM + CO_2) increased linearly from 0–3 h (Figure 1A). Adding AICA riboside decreased ^{14}C labelling of TAG at 1 and 3 h by 39 % and 50 % respectively ($P < 0.001$) (Figure 1A), and decreased ^{14}C labelling of DAG by 29 % and 60 % ($P < 0.001$) (Figure 1B). AICA riboside did not affect [^{14}C]oleate incorporation into phospholipid (Figure 1A) or cholesterol esters (Figure 1B). Similarly, AICA riboside decreased [^{3}H]glycerol incorporation into TAG at 1 and 3 h by 47 % and 38 % ($P < 0.001$) respectively, but did not affect ^{3}H labelling of phospholipid (Figure 1C). These data suggest that AMPK decreases *de novo* the synthesis of TAG and its immediate precursor DAG, but without affecting the synthesis of phospholipid or cholesterol esters. The observation that AICA riboside neither affected cell viability for up to 6 h (results not shown) nor decreased all end products provides evidence against a generalized effect of AICA riboside that would compromise cell function.

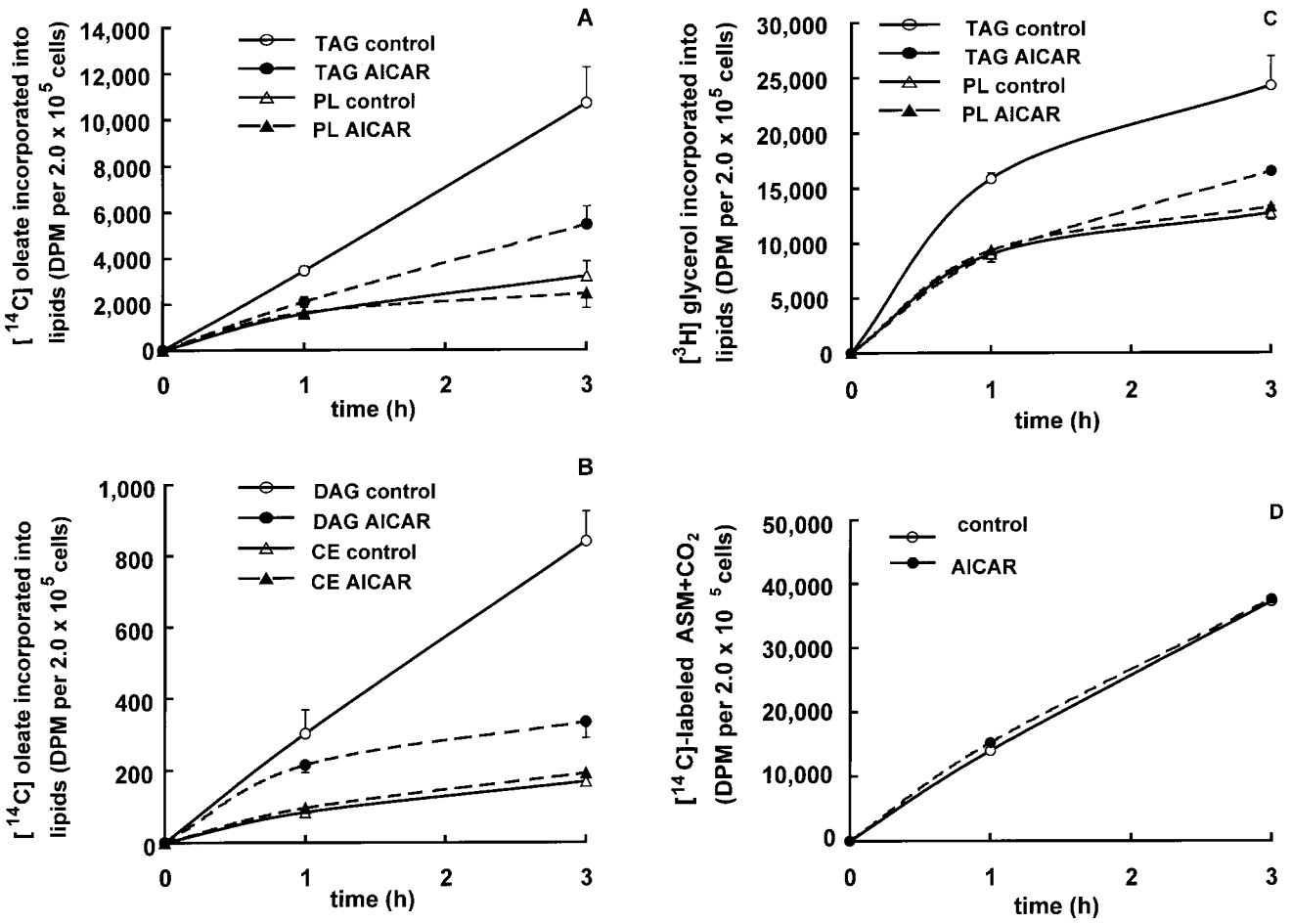


Figure 1 Effect of AICA riboside on hepatocyte cell cultures

Hepatocyte monolayers were incubated at 37 °C with 0.2 mM [^{14}C]oleate for 1–3 h with or without 0.5 mM AICA riboside (AICAR). Incorporation of [^{14}C]oleate was measured into (**A**) TAG and phospholipids (PL), (**B**) DAG and cholesteryl ester (CE), and (**D**) oxidation products (ASM + CO_2). (**C**) Hepatocyte monolayers were incubated with 0.2 mM oleate and 0.5 mM [^{3}H]glycerol for 1–3 h with or without 0.5 mM AICAR, and incorporation of [^{3}H]glycerol was measured into TAG and PL. Values represent means ± S.D. for three to six wells, each collected during three separate incubations.

Because the activities of TAG-biosynthetic enzymes might decrease with time in cultured hepatocytes, we also tested the effect of AICA riboside on suspensions of freshly isolated hepatocytes. At 1 h, AICA riboside decreased [^{14}C]oleate incorporation into TAG, DAG and phospholipid by 47 %, 96 % and 45 % ($P < 0.001$) respectively, but did not inhibit labelling of cholesterol esters (results not shown). The observation that AICA riboside's effects on DAG and phospholipid were greater in freshly isolated hepatocytes than in cultured cells might be related to hepatocyte de-differentiation and down-regulation of liver-specific and lipogenic enzymes in cultured hepatocytes. Interestingly, [^{14}C]oleate incorporation into cholesterol esters reached a plateau by 1 h in control cells, but remained linear up to 2 h in AICA riboside-treated cells, so that, at 2 h, AICA riboside increased ^{14}C labelling of cholesterol ester by 77 % ($P < 0.001$). This observation suggested the possibility that in control cells, the rates of cholesterol ester synthesis and hydrolysis were equal during the second hour of incubation, and that AMPK might inhibit the hydrolysis of cholesterol esters (as discussed below).

AICA riboside did not alter the labelling of oxidation products in hepatocyte suspensions (results not shown). This differs from a hepatocyte study in which a 10 min incubation with AICA riboside stimulated long-chain fatty acid oxidation [8]. The differences between our data and those reported previously is likely to result from our longer incubation time with [^{14}C]oleate. During longer [^{14}C]oleate incubations, more of the hepatocyte TAG pool becomes labelled, but because AICA riboside decreases [^{14}C]oleate incorporation, the specific activity of the TAG pool is lower when AICA riboside is present. Because a large portion of fatty acid oxidized by hepatocytes is derived from the lipolysis of intracellular TAG (results not shown), a decrease in TAG-specific activity would mask subsequent stimulation by AICA riboside of fatty acid entry into mitochondria. When the incubation is longer, there may be no observable effect on net [^{14}C]oleate oxidation.

AMPK regulates glycerolipid metabolism in isolated skeletal muscle

Because AMPK regulates fatty acid oxidation in skeletal muscle [9], we investigated the effects of AICA riboside in isolated mouse soleus muscles. During a 90 min incubation, 1.0 mM AICA riboside increased [^{14}C]oleate oxidation to CO_2 by 48 %

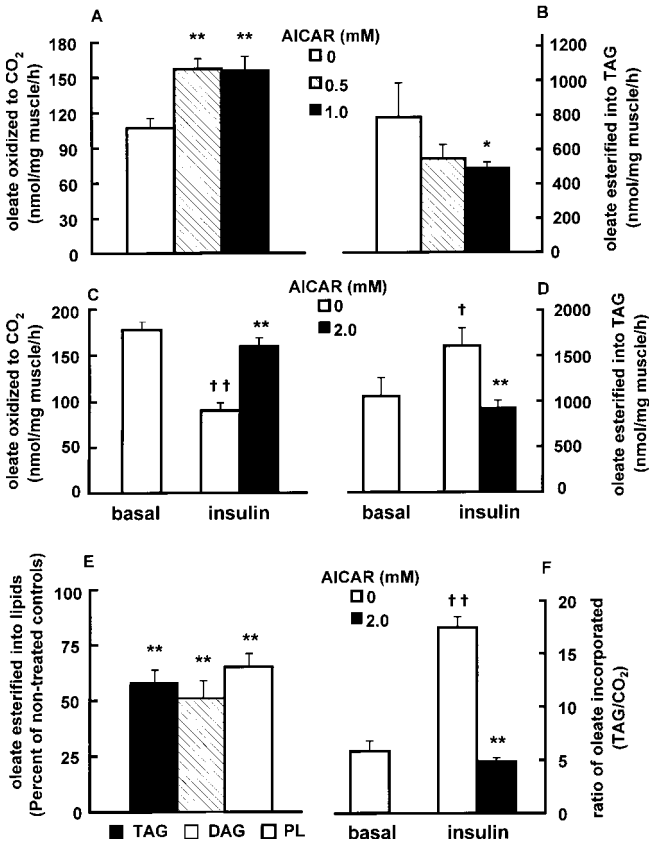


Figure 2 Effect of AICA riboside in isolated soleus muscles

Mouse soleus muscles were incubated with 1.0 mM [^{14}C]oleate for 90 min at 37 °C with 0, 0.5 or 1.0 mM AICA riboside (AICAR). Incorporation of [^{14}C]oleate into (**A**) CO_2 and (**B**) TAG was determined. Muscles were incubated for 90 min with 1.5 mM [^{14}C]oleate plus 10^{-7} M insulin, with or without 2.0 mM AICA riboside, and [^{14}C]oleate incorporation into (**C**) CO_2, (**D**) TAG and (**E**) DAG and phospholipids (PL) was determined. (**F**) Fatty acid partitioning was quantified for each muscle by dividing the amount of ^{14}C label recovered in TAG by the amount of [^{14}C]oleate oxidized to CO_2. Values represent means ± S.E.M. for four to six muscles. Differences between AICA riboside-treated muscles and contralateral controls were analysed by paired Student's *t* test ($^{*}P < 0.05$; $^{**}P < 0.005$). Differences between controls and insulin-treated muscles were analysed by single-factor analysis of variance ($^{\dagger}P < 0.05$; $^{\dagger\dagger}P < 0.005$).

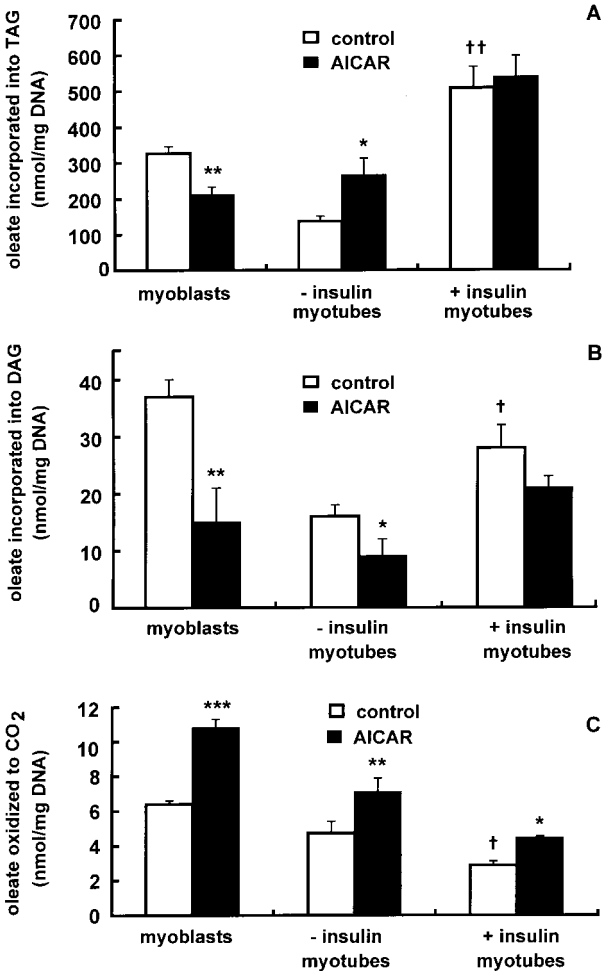


Figure 3 Effect of AICA riboside in muscle cell cultures

On day 0 (myoblasts) and day 5 (myotubes) in differentiation media, myocytes were incubated for 2 h in 0.25 mM [^{14}C]oleate, with or without 1.0 mM AICA riboside (AICAR) and 10^{-7} M insulin. [^{14}C]Oleate incorporation into (**A**) TAG, (**B**) DAG and (**C**) CO_2 was determined. Values represent means ± S.D. for three to four wells collected during three separate incubations. Differences between AICA riboside-treated and control cells were detected by Student's *t* test ($^{*}P < 0.05$; $^{**}P < 0.01$). Differences between insulin-treated groups were analysed by single-factor analysis of variance ($^{\dagger}P < 0.05$; $^{\dagger\dagger}P < 0.005$).

(Figure 2A) and decreased [^{14}C]oleate incorporation into TAG by 37 % (Figure 2B).

In order to investigate the effects of AICA riboside during incubation conditions that normally promote lipogenesis, and to determine whether AICA riboside blocks the lipogenic action of insulin, we incubated soleus muscles in media containing 10^{-7} M insulin and 1.5 mM [^{14}C]oleate. Consistent with our previous observations [25], adding insulin to the media decreased oleate oxidation by 49 % and increased oleate incorporation into TAG by 62 % (Figures 2C and 2D respectively). Adding AICA riboside completely blocked both the antioxidative and the lipogenic effects of insulin; fatty acid oxidation increased by 78 %, fatty acid labelling of TAG decreased by 43 %, and fatty acid esterification into DAG and phospholipid decreased by 49 % and 35 % respectively (Figure 2E). The combined effects of AICA riboside on fatty acid oxidation and fatty acid esterification resulted in a 71 % decrease in the ratio of fatty acid esterified into TAG/fatty acid oxidized (Figure 2F). These data indicate that AICA riboside alters muscle fatty acid partitioning between oxidative and lipogenic pathways, and that AMPK-mediated regulation of fatty acid oxidation and glycerolipid metabolism are reciprocally co-ordinated.

AMPK regulates glycerolipid metabolism in cultured skeletal muscle cells

To establish a muscle cell model in which to study the mechanism of AICA riboside-induced regulation of muscle glycerolipid metabolism, we studied mouse C_2C_{12} myocytes, which can be induced to differentiate from myoblasts into myotubes [26,27]. Although myotubes incorporated 58 % less [^{14}C]oleate into TAG than did undifferentiated myoblasts (Figure 3A), at both stages of differentiation AICA riboside increased oleate oxidation by 50–70 % (Figure 3C). In undifferentiated myoblasts, AICA riboside decreased [^{14}C]oleate incorporation into TAG by 35 %, DAG by 59 % (Figures 3A and 3B respectively) and phospholipid by 26 % [for control, 435 ± 45 nmol/mg DNA, and with AICA

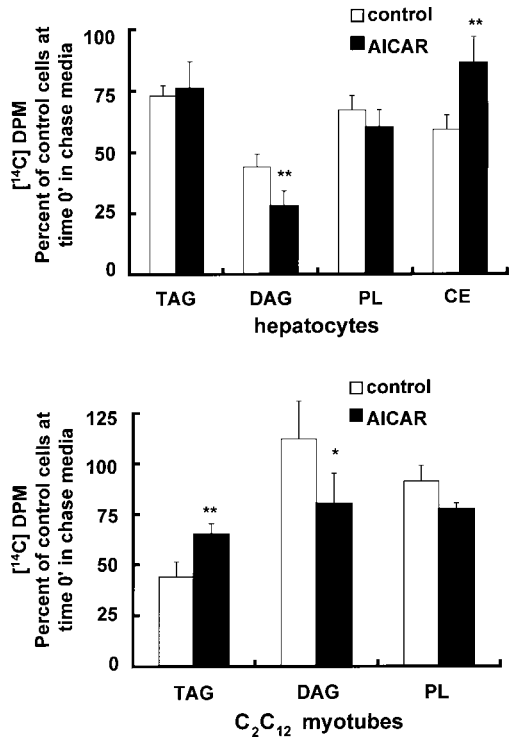


Figure 4 Effect of AICA riboside on lipid hydrolysis in hepatocytes and muscle cell cultures

(**A**) After hepatocyte monolayers were labelled for 12 h with 0.2 mM [^{14}C]oleate, they were incubated at 37 °C for 3 h in chase media containing 0.2 mM oleate with or without 0.5 mM AICA riboside (AICAR), and ^{14}C label incorporation into TAG, DAG, phospholipids (PL) and cholesteryl ester (CE) was measured. Data are expressed as a percentage of the label lipids recovered from cells collected immediately after the 12 h labelling period (in units of d.p.m./2.0×10^5 cells: TAG, 5359 ± 586; DAG, 230 ± 35; PL, 4855 ± 679; CE, 552 ± 67). (**B**) After myotubes (day 6 in differentiation media) were labelled for 3 h with 0.25 mM [^{14}C]oleate, they were incubated for 3 h in chase media containing 0.25 mM oleate with or without 1.0 mM AICAR. [^{14}C]oleate incorporation into TAG, DAG and PL was measured. Data are expressed as a percentage of the label lipids recovered from myotubes collected immediately after the 3 h preincubation (in units of d.p.m./μg DNA: TAG, 1400 ± 113; DAG, 130 ± 18; PL, 2731 ± 226). Values represent means $\pm$ S.D. for six wells. Differences between groups were detected by Student's t test (*$P < 0.05$; **$P < 0.001$).

riboside, 318 ± 45 nmol/mg DNA ($P < 0.005$)]. Although AICA riboside decreased labelling of DAG by 44 % in differentiated myotubes, oleate incorporation into TAG increased by 93 %. When insulin was present, oleate incorporation into TAG increased by 270 % compared with controls in the absence of insulin, and AICA riboside had no additional effect.

Effect of AICA riboside on hepatocyte and myocyte lipolysis

Others have reported that AMPK phosphorylates and inactivates hormone-sensitive lipase, thereby decreasing adipocyte lipolysis [10]. Hormone-sensitive lipase is also expressed in muscle, but lipolysis in hepatocytes is catalysed by different lipases. Our observations that AICA riboside increased ^{14}C labelling of both TAG in C_2C_{12} myotubes and cholesterol esters in hepatocyte suspensions suggested that AMPK might inhibit the hydrolysis of TAG and cholesterol esters. To test this prediction, we labelled hepatocyte and C_2C_{12} myotube lipids with [^{14}C]oleate, and then examined the effects of AICA riboside on lipid hydrolysis. In hepatocytes, 27 % of the labelled TAG was hydrolysed during the 3 h chase (Figure 4A). Adding AICA riboside to the chase media decreased [^{14}C]DAG by 37 % and increased [^{14}C]cholesterol ester by 46 %, but had no effect on labelling of TAG or phospholipids. These data indicate that the AICA riboside-induced decreases in hepatocyte [^{14}C]TAG levels (above) were due to decreased TAG biosynthesis. Furthermore, the data also suggest that, in hepatocytes, distinct lipases hydrolyse TAG and cholesterol esters, and AMPK regulates only the lipase that hydrolyses cholesterol esters. In C_2C_{12} myotubes, 66 % of [^{14}C]TAG was hydrolysed during the 3 h chase and AICA riboside inhibited TAG lipolysis by 47 % (Figure 4B). The decrease in myocyte [^{14}C]TAG in the control compared with AICA riboside-treated cells was quantitatively accounted for by a 21 % increase ($P < 0.01$) in the amount of ^{14}C-label recovered from the chase media. Similarly to our observation in hepatocytes, AICA riboside in the chase media decreased myotube [^{14}C[DAG by 29 %, but had no effect on the labelled phospholipids.

AMPK inhibits GPAT activity in hepatocytes

In each of the cell or tissue types studied, AICA riboside significantly decreased labelling of the lipid intermediate, DAG. A decrease in labelled DAG could occur either by inactivating one of the first steps in the pathway of DAG synthesis, or by rapid use of DAG to synthesize TAG and phospholipid. Because AICA riboside decreased TAG and, under certain conditions, phospholipid synthesis, our data suggested that AICA riboside inhibits the *de novo* pathway of glycerolipid synthesis. Furthermore, the AMPK-mediated increase in fatty acid oxidation could account for only a small percentage of the absolute decrease in the amount of fatty acid esterified into complex lipids. Our observation that AICA riboside inhibits fatty acid incorporation into TAG, DAG and phospholipid, but not cholesterol esters, suggests that AMPK might inhibit at an early step in glycerolipid synthesis. The most probable target for inhibition is the enzyme that catalyses the first and committed step in glycerolipid biosynthesis, GPAT. Mammalian cells contain two GPAT isoenzymes, one located in the outer mitochondrial membrane and the other in the endoplasmic reticulum [17]. The mitochondrial isoenzyme comprises 30–50 % of liver GPAT activity, but less than 10 % of GPAT activity in other tissues [17]. The activities of these two isoenzymes can be distinguished because only the endoplasmic reticulum (microsomal) GPAT is inactivated by NEM.

To determine whether AMPK inactivates the two GPAT activities, we used hepatocytes. When hepatocytes are isolated by collagenase perfusion, AMPK activity is initially very high, but declines by 80 % during a 60 min incubation [2]. Similarly, the activities of AMPK substrates, ACC and HMG-CoA reductase, are initially low and increase during this time. Therefore, to determine whether similar changes occur for GPAT, we measured GPAT activity in freshly isolated hepatocytes 30 min before adding AICA riboside, and during the 60 min after adding AICA riboside (Figure 5). In control cells, activities of mitochondrial and microsomal GPAT isozymes increased 44 % and 53 % respectively during the 90 min experiment. Incubating cells with AICA riboside decreased mitochondrial GPAT activity by 29–43 % in a time-dependent manner (Figure 5A). The effect of AICA riboside on mitochondrial GPAT was most pronounced at 40 min, after which the activity in control cells also declined. During the first 40 min after adding AICA riboside, microsomal GPAT activity was unaffected (Figure 5B). However, after a 60 min incubation with AICA riboside, microsomal GPAT decreased 35 %. From these data, it appears that AMPK might regulate both GPAT isoforms, but that inactivation of the

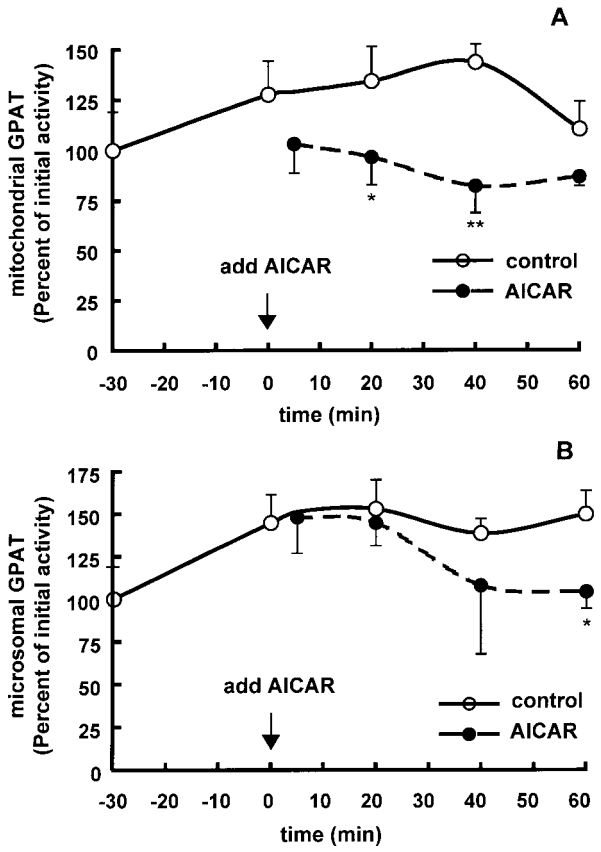


Figure 5 Effect of AICA riboside on GPAT activities in isolated hepatocytes

Mitochondrial (**A**) and microsomal (**B**) GPAT activities were measured in total particulate preparations isolated from hepatocytes incubated for 0–90 min. After a 30 min preincubation, AICA riboside (AICAR; 0.5 mM) was added to the incubation media (indicated by downward arrows). Data are expressed as a percentage of the initial enzyme activities measured in hepatocytes that were collected and frozen immediately after isolating the cells. Initial activities were 0.233 ± 0.045 nmol/mg per min and 0.547 ± 0.064 nmol/mg per min for mitochondrial and microsomal GPAT respectively. Values represent means $\pm$ S.D. for three to six wells collected during two separate incubations. $^{*}P < 0.01$; $^{**}P < 0.001$.

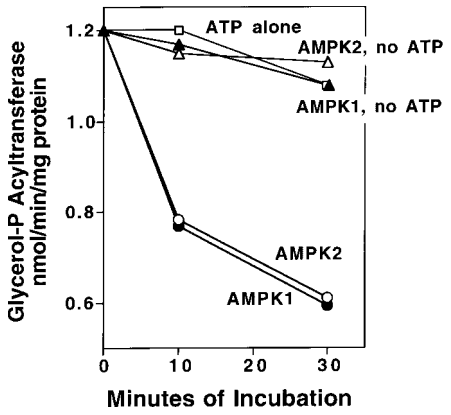


Figure 6 Inhibition of mitochondrial GPAT activity by AMPK

Rat liver mitochondria (150 μg of protein) were incubated with AMPK1 or AMPK2 with or without 1.3 mM ATP. After 10 or 30 min, aliquots were assayed for GPAT activity at 20, 30 and 40 μg of mitochondrial protein, as described in the Experimental section. Data are shown from an experiment that was repeated three additional times with inhibitions ranging from 30–50%.

mitochondrial enzyme occurs more rapidly. These results were corroborated by two additional experiments in which exposing hepatocytes to 0.5 mM AICA riboside for both 15 and 30 min decreased mitochondrial GPAT by 22–34%, without affecting microsomal GPAT activity (results not shown).

Since AMPK inhibits ACC activity and consequently decreases liver concentrations of malonyl-CoA, we investigated the possibility that malonyl-CoA is a regulator of GPAT. Adding concentrations of malonyl-CoA of 0.5–10.0 mM affected neither mitochondrial nor microsomal GPAT activity (results not shown).

To determine whether the effect of AICA riboside was similar in skeletal muscle, we measured GPAT activity in C_2C_{12} myocytes. In myotubes, microsomal GPAT activity was 0.51 ± 0.06 nmol/mg per min, and was unaffected by a 1 h incubation with AICA riboside. Because mitochondrial GPAT activity accounted for only 10% of the low total GPAT activity in these cells, it could not be measured reliably. The low mitochondrial GPAT activity usually found in cultured cell lines [17,28] might partly explain the observed differences in AICA riboside's effects on glycerolipid metabolism in hepatocytes compared with myocytes.

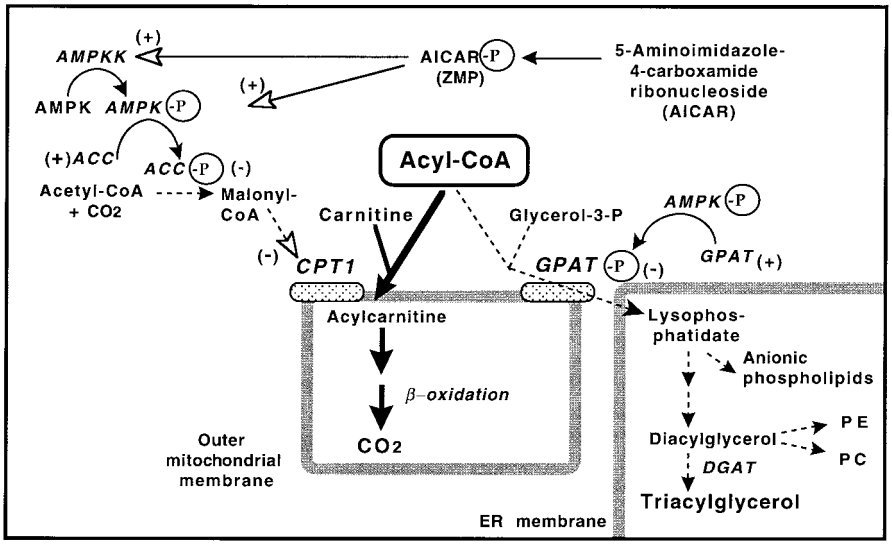
In order to determine the specificity of the effects of AICA riboside, we measured the activities of DGAT, the activity unique to TAG synthesis, and ACS, the activity required to activate fatty acids for metabolism by both the oxidative and synthetic pathways. In the same hepatocyte membrane preparations in which GPAT assays had been performed, activities of DGAT and ACS were 1.06 ± 0.25 and 9.82 ± 0.61 nmol/mg per min respectively. Incubating cells for 5–60 min with 0.5 mM AICA riboside had no effect on the activity of either DGAT or ACS.

AMPK1 and AMPK2 inhibit mitochondrial GPAT activity

Recombinant AMPK1 and AMPK2 both inhibited liver mitochondrial GPAT 21% after 10 min and 40% after 30 min of incubation (Figure 6). The amount of inhibition was identical for AMPK1 and AMPK2. Inhibition was dependent on both time and the presence of ATP. ATP alone or boiled AMPK had no effect (results not shown). In a microsomal membrane preparation that contained 12% NEM-resistant (mitochondrial) GPAT, a 30 min incubation with AMPK inhibited approx. 4% of the activity, consistent with inhibition of only the mitochondrial GPAT isoform (results not shown).

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 pathways that use energy, and stimulating pathways that produce energy [1]. In hepatocytes [29], adipocytes [10], myocytes [6] and perfused muscle [9], AICA riboside, at the concentrations used in our experiments, stimulates AMPK activity up to 12-fold without changing cellular concentrations of ATP, AMP or ADP. We used AICA riboside to provide evidence that AMPK inhibits the synthesis of TAG, DAG and phospholipid. In both hepatocytes and isolated skeletal muscle, AICA riboside decreased [^{14}C]oleate incorporation into TAG, DAG and phospholipid by 45–95% without inhibiting label incorporation into cholesterol esters. AICA riboside also decreased [^{3}H]glycerol incorporation into these glycerolipids, suggesting that AMPK mediates inhibition



Scheme 1 Reciprocal regulation of fatty acid oxidation and TAG biosynthesis by AMPK

Within the cell, AICA ribonucleoside (AICAR) is phosphorylated to form ZMP, an AMP analogue that activates both AMPK and AMPK kinase (AMPKK). Activated AMPK phosphorylates and inactivates ACC, thereby decreasing malonyl-CoA levels and relieving CPT1 inhibition. AMPK also phosphorylates and inactivates mitochondrial GPAT so that less lysophosphatidic acid is synthesized. Both GPAT and CPT1 are intrinsic membrane proteins (shown schematically by oblong dot-filled boxes) whose active sites face the cytosolic surface of the outer mitochondrial membrane and can compete for acyl-CoAs. The net effect of AMPK activation is to channel acyl-CoAs towards β-oxidation and away from glycerolipid biosynthesis. Plus or minus signs in parentheses indicate activatory or inhibitory effects respectively. ER, endoplasmic reticulum; PC, phosphatidylcholine; PE, phosphatidylethanolamine.

of lipid synthesis *de novo* at an early step in the pathway of glycerolipid biosynthesis.

De novo synthesis of TAG and phospholipid involves a complex multienzyme pathway with several common intermediates, including DAG (Scheme 1). Since GPAT catalyses the first and committed step in glycerolipid synthesis, and is believed to be rate-limiting, it represents an obvious site for the regulation of fatty acid esterification. Adding AICA riboside to rat hepatocyte suspensions inhibited the activity of the mitochondrial, but not the microsomal, GPAT activity. Furthermore, in isolated mitochondria, purified AMPK containing the α-1 or α-2 catalytic subunits inhibited mitochondrial GPAT in both a time- and ATP-dependent manner. These data suggest that the mitochondrial GPAT might be phosphorylated and inactivated by AMPK. Indirect evidence from other laboratories had suggested that mitochondrial GPAT is acutely regulated, and that inactivation is associated with conditions that would favour GPAT phosphorylation [30]. Analysis of the rat mitochondrial GPAT cDNA sequence predicts four potential phosphorylation sites that match the consensus motif recognized by AMPK (NCBI PDB 2444459). Whether AMPK inhibits mitochondrial GPAT by directly phosphorylating the enzyme or by phosphorylating an intermediate regulatory protein remains to be determined.

AICA riboside's effect as an activator of AMPK has been well characterized in hepatocytes, and appears to be independent of changes in other purine nucleotides [10,29]. Although small amounts of the triphosphorylated derivative, ZTP, are formed in hepatocytes, the metabolic consequences of increased cellular ZTP are unknown, and, at 15 min when activation of AMPK is maximal, the concentration of ZTP in hepatocytes is very low (13 %) compared with ATP [10]. Thus, since we observed AICA riboside-mediated decreases in mitochondrial GPAT at early time points, it is unlikely that the effect was non-specific and related to ZTP accumulation. Our data showing that purified AMPK inhibits mitochondrial GPAT activity strongly suggest that AICA riboside's effect to decrease TAG synthesis in muscle and liver is due to AMPK-mediated inactivation of mitochondrial GPAT. However, since AICA riboside also mimics the effects of AMP on other energy-regulating enzymes, such as glycogen phosphorylase [5] and fructose-1,6-bisphosphatase [31], we cannot rule out the possibility that AICA riboside might also affect TAG synthesis indirectly via mechanisms that are independent of AMPK.

Because the specific activity of GPAT in skeletal muscle is very low, we were unable to test directly the effects of AICA riboside or AMPK on muscle GPAT activities. However, our study of [^{14}C]oleate partitioning in muscle implies that AICA riboside's effects are similar in muscle and liver. In isolated soleus muscle, AICA riboside increased [^{14}C]oleate oxidation by 48–78 %. This increase corroborates a study in perfused rat hind-limb in which AICA riboside-induced activation of AMPK stimulated fatty acid oxidation 2.8-fold, together with a 75 % decrease in muscle ACC activity and a 50 % decrease in malonyl-CoA [9]. To quantify the net effect of AICA riboside on acyl-CoA partitioning, we divided the amount of fatty acid esterified into TAG by the amount of fatty acid oxidized to CO_2. When insulin was present in the incubation media to promote lipogenesis, 15 times more [^{14}C]oleate was esterified into TAG than was oxidized. Adding AICA riboside to the media decreased this ratio by 78 %, clearly illustrating the marked effect of AMPK on fatty acid partitioning between the oxidative and biosynthetic pathways. These observations might have important physiological relevance during endurance exercise, which stimulates muscle AMPK activity and shifts muscle-substrate utilization in favour of fatty acids.

Insulin-mediated suppression of muscle fatty acid oxidation is believed to result from stimulation of ACC activity, thereby increasing the malonyl-CoA concentration which would inhibit CPT1 and, thus, fatty acid transport into mitochondria [32]. Although it is yet unclear how insulin increases fatty acid

incorporation into TAG, in perfused rat liver, cardiomyocytes and cultured adipocytes, insulin acutely activates GPAT [33–35]. In most of these studies, however, the mitochondrial and microsomal isoenzymes were not differentiated. Because AICA riboside and insulin had opposite effects on fatty acid partitioning in C_2C_{12} myocytes and isolated soleus muscle, AMPK might counteract insulin's action on muscle fatty acid partitioning via their common targets, ACC and mitochondrial GPAT. Differences between myocytes and isolated soleus muscle in their incorporation of fatty acid into TAG during incubation with AICA riboside might be due to differences in the expressed activities of GPAT, CPT1 and/or other enzymes, such as TAG lipase, that determine muscle lipid metabolism.

Mitochondrial GPAT is developmentally, nutritionally and hormonally regulated [36,37], but its precise role remains elusive because mitochondria lack the enzymic machinery necessary to synthesize glycerolipids beyond lysophosphatidic acid (Scheme 1) [30,38]. Thus lysophosphatidate must be transported from the mitochondria to the endoplasmic reticulum, where TAG and phospholipid biosynthesis occur [39]. Since mitochondrial GPAT and CPT1 are both located on the outer mitochondrial membrane, the mitochondrial isoform of GPAT is uniquely positioned to compete directly with CPT1 for acyl-CoA substrates. It is therefore tempting to speculate that, by converting acyl-CoA into lysophophatidate, mitochondrial GPAT plays a pivotal role in preventing fatty acids from undergoing β-oxidation. Regulation of the relative activities of mitochondrial GPAT and CPT1 would thus control the branch point at which acyl-CoA substrates are partitioned towards either oxidative or biosynthetic pathways. We propose that when cellular ATP levels are compromised, AMPK-mediated inactivation of mitochondrial GPAT serves not only to conserve energy by inhibiting lipid biosynthesis, but, by eliminating CPT1's direct competitor for acyl-CoA substrates, also potentiates fatty acid entry into mitochondria.

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