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Inhibition of lipolysis and lipogenesis in isolated rat adipocytes with AICAR, a cell-permeable activator of AMP-activated protein kinase

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Abstract In vivo, hormone-sensitive lipase (HSL) is known to be phosphorylated on two sites termed the regulatory and basal sites. However, the intracellular role of the basal site or the identity of the protein kinase phosphorylating this site has not been established. We show that 5-amino-4-imidazolecarboxamide ribonucleoside (AICAR) markedly activates cellular AMP-activated protein kinase (AMPK) in a time- and dose-dependent manner. As expected for an agent that activates AMPK intracellularly, AICAR had no effect on the basal activity of HSL. However, preincubation of adipocytes with AICAR led to a reduced response of these cells to the lipolytic agent isoprenaline. AICAR was also shown to profoundly inhibit lipogenesis through increased phosphorylation of acetyl-CoA carboxylase (ACC). Thus it appears that in addition to regulating lipogenesis, AMPK also plays an important antilipolytic role by regulating HSL in rat adipocytes.

Key words: AMP-activated protein kinase; Hormone-sensitive lipase; Acetyl-CoA carboxylase; Lipolysis; Lipogenesis; Adipocyte (rat)

1. Introduction

Hormone-sensitive lipase (HSL) is the rate-limiting enzyme for the mobilisation of free fatty acids from adipose tissue and acetyl CoA carboxylase (ACC) is the key enzyme involved in the de novo biosynthesis of long-chain fatty acids. Both enzymes are regulated by acute and long term mechanisms, with the latter involving changes in gene expression [1–3].

Short-term regulation of the activity of ACC involves complex allosteric modulation and reversible protein phosphorylation [4]. Experimental data from several studies with intact cells and in vivo have shown that the increased phosphorylation and concomitant inactivation of ACC in response to both hormones and dietary manipulations is regulated by AMPK [5,6]. Furthermore, in rat hepatocytes and Fao hepatoma cells, it has been demonstrated that conditions which raise intracellular 5′-AMP levels, e.g. treatment with ATP depletors [7], fructose [8], heat shock or arsenite [9], cause a transient but marked activation of AMPK leading to concomitant inhibition of ACC and consequently fatty acid biosynthesis [7–9].

The hydrolysis of triacylglycerol by HSL is tightly controlled by a variety of hormonal and neural signals. Lipolytic agents such as adrenaline and isoprenaline acutely regulate HSL by raising cAMP levels to cause activation of cAMP-dependent protein kinase (PKA) which in turn phosphorylates and activates HSL leading to the stimulation of lipolysis [10,11]. PKA has been shown to phosphorylate a single site on HSL in vitro [12,13] and additional studies have shown that this site, termed the regulatory site, is also phosphorylated in primary rat adipocytes in response to lipolytic agents [12]. A second site, termed the basal site, has also been shown to be partially phosphorylated in purified preparations of the enzyme suggesting that this may also occur in vivo [12]. Although a number of protein kinases phosphorylate the basal site in vitro, they have no direct effect on the activity of HSL. However, recent work has shown that in vitro phosphorylation of the basal site by AMPK, Ca^{2+}/calmodulin-dependent protein kinase or glycogen synthase kinase 4 prevents phosphorylation and subsequent activation of HSL by PKA [14]. On the basis of these in vitro data, it has been suggested that phosphorylation of the basal site plays a physiological role in the regulation of lipolysis [14]. However, to date, there has been no progress to determine whether this mechanism operates in vivo and if so, which protein kinase(s) is involved.

Clearly, one approach to determine which protein kinase(s) is important in the phosphorylation of the basal site within HSL in vivo is to identify agents that activate these kinases in primary adipocytes and examine their effects on lipolysis. Recently, we identified a number of 5′-AMP analogues that activate rat and human liver AMPKs in vitro [15]. In this study we show that 5-amino-4-imidazolecarboxamide ribonucleoside (AICAR), the cell-permeable precursor of 5-amino-4-imidazolecarboxamide ribotide (ZMP) [16], activates AMPK in primary adipocytes resulting in the inhibition of lipolysis and lipogenesis.

2. Materials and methods

Collagenase was purchased from Worthington Biochemical Corporation (UK). [γ-^{33}P]ATP, [^{14}C]bicarbonate and [3-^{3}H]glucose were purchased from Amersham International. Optiscint Hisafe was purchased from Wallac (Milton Keynes, UK). All other reagents were purchased from Sigma Chemical Co (UK) unless otherwise indicated.

2.1. Isolation of primary rat adipocytes

Isolated adipocytes were obtained from the epididymal fat pads of fed rats (Alderley Park, 150–200 g) by collagenase digestion [17]. Aliquots of fat cells (1×10^6 cells/ml) were incubated in polyethylene tubes

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Abbreviations: AMPK, AMP-activated protein kinase; AICAR, 5-amino-4-imidazolecarboxamide ribonucleoside; HSL, hormone-sensitive lipase; ACC, acetyl-CoA carboxylase; PKA, cAMP-dependent protein kinase; OkA, okadaic acid.

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in Krebs–Ringer–HEPES buffer (pH 7.4) supplemented with 1% (w/v) BSA at 37°C and agitated at 65 rpm for 1 h prior to use.

2.2. AMPK and ACC assays

Triplicate aliquots of adipocytes were incubated for 1 h (37°C, 65 rpm) in polyethylene tubes in Krebs–Ringer–HEPES buffer (pH 7.4) supplemented with 1% (w/v) BSA with or without AICAR (as indicated in the figure legends). The cells were separated from the media by flotation, resuspended in Buffer A (pH 7.2 at 4°C 100 mM Tris-HCl, 250 mM Mannitol, 1 mM EDTA, 50 mM NaF, 5 mM sodium pyrophosphate, 1 mM DTT, 1 mM benzamidine, 5μg/ml trypsin inhibitor) and immediately frozen in liquid nitrogen. Upon defrosting, the cellular debris was pelleted by centrifuging at 13,000 r.p.m for 3 min and the resulting supernatant removed and adjusted to 6% with polyethylene glycol (PEG). Following further centrifugation for 5 min (13,000 rpm), the pellet was resuspended in Buffer A. Aliquots were used to assay the AMPK activity by the SAMS peptide phosphorylation assay in the presence of saturating (200 μM) concentrations of 5′-AMP as previously described [15]. The initial activity of ACC was assayed by measuring the incorporation of [^{14}C]bicarbonate into malonyl-CoA [18]. Each reaction mixture contained 100 mM Tris-HCl pH 7.4, 0.3 mM acetyl-CoA, 4 mM ATP, 2 mM $MgCl_2$ 10 mg/ml bovine serum albumin, 15 mM Na[^{14}C]HCO_3 (2 Ci/mol) and an aliquot of the resuspended PEG pellet fraction containing ACC.

Trisodium citrate and equimolar $MgCl_2$ were at 10 mM. Assays were terminated after 5 min at 37°C using 5 M HCl. Tubes were centrifuged at 13,000 r.p.m. for 2 min and an aliquot of the supernatant was evaporated to dryness. Residues were dissolved in H_2O and assayed for radioactivity in PCS scintillation fluid. For total activity measurements, ACC was prepared in Buffer A lacking NaF and sodium pyrophosphate and incubated for 30 min at 37°C prior to assay.

2.3. Measurement of lipolysis

Triplicate aliquots of adipocytes were incubated with AICAR for 1 h (37°C, 65 rpm) as in section 2.2. Following incubation, the cells were then challenged with isoprenaline (10^{-9} to 10^{-5}M) in 0.3 mM ascorbate for 30 min (37°C, 65 rpm). An aliquot of medium was removed and assayed for glycerol using Triglyceride Reagent A. Concentrations of glycerol were determined spectrophotometrically at a wavelength of 590 nm with reference to a standard calibration curve over the range 1–125 mg/dl.

2.4. Measurement of lipogenesis

Lipogenesis was measured according to the method of Moody et al. [19]. Triplicate aliquots of adipocytes were preincubated for 30 min (conditions described in section 2.1) in the presence of 1 nM insulin and then for a further 60 min in the absence or presence of AICAR and radiolabelled glucose (0.5 mM, 0.6 Ci/mol). Reactions were terminated by addition of Optiscint fluid, mixed thoroughly and left to settle for 60 min. An aliquot of the top phase was removed and counted for incorporation of tritiated glucose into total lipid.

2.5. Other methods and analysis

The SAMS peptide was synthesised as described previously [15]. Kinetic parameters were analysed by non-linear regression using Graph-Fit software.

3. Results

3.1. Activation of adipocyte AMP kinase

We have recently shown that ZMP can mimic 5′-AMP by allosterically activating both rat and human liver AMPK in vitro [15]. The results presented here show that rat adipocyte AMPK is also activated by both 5′-AMP and ZMP. With 5′-AMP, the V_{max} was 76 ± 5 pmol/min/ 10^6 cells with a K_m for the peptide of 35μM. With ZMP, the V_{max} was 55 ± 3 pmol/min/10^6 cells with a K_m of 103 μM. The kinetic constants show that ZMP is a weaker activator of adipocyte AMPK compared to 5′-AMP (V_{max}/K_m ~ 4-fold lower).

To determine the intracellular effects of ZMP on AMPK activity, we used the cell permeable nucleoside precursor of

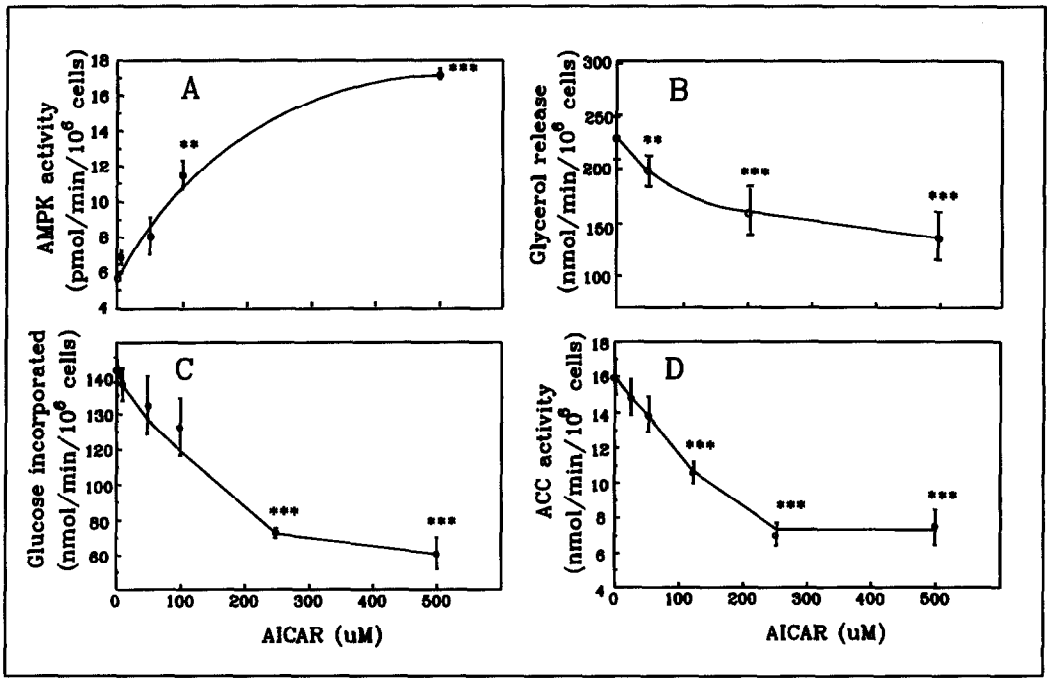

Fig. 1. Effects of a dose response to AICAR in primary adipocytes. (Panel A) Activation of PEG-purified AMPK by AICAR expressed as pmol ^{33}P incorporated into the SAMS peptide/min/million cells. Samples were assayed in the presence of saturating concentrations of 5′-AMP (200 μM). (Panel B) Inhibition of lipolysis by AICAR expressed as nmol glycerol released/min/million cells. (Panel C) Inhibition of lipogenesis by AICAR expressed as nmol glucose incorporated into total lipid/min/million cells. (Panel D) Inhibition of ACC activity by AICAR expressed as nmol [^{14}C]bicarbonate incorporated into malonyl CoA/min/million cells. In all experiments the data points represent the mean of three separate cell incubations. The standard deviations from the mean are indicated by the vertical bars. The statistical significance of differences, as determined by a two-tailed *t*-test, is indicated (***$P < 0.001$, **$P < 0.002$).

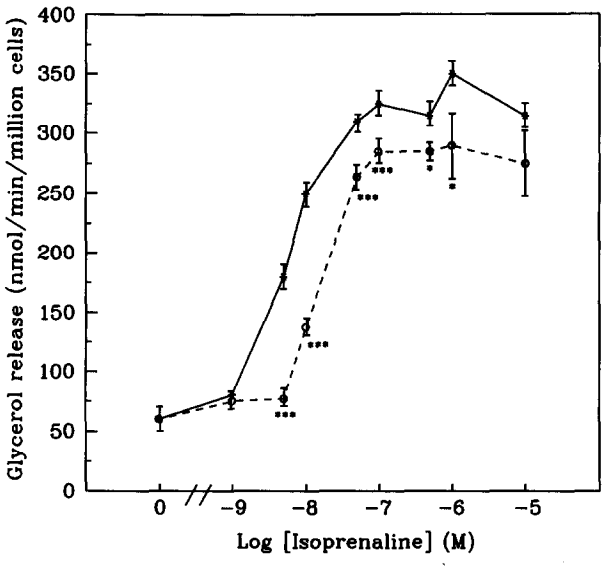

Fig. 2. Effect of AICAR on lipolysis stimulated by isoprenaline in primary adipocytes. Adipocytes were incubated for 60 min in control experiments (*) or with 500 μM AICAR (○) prior to a challenge with isoprenaline. The data points represent the mean of three separate cell incubations. The standard deviations from the mean are indicated by the vertical bars. The statistical significance of differences, as determined by a two-tailed *t*-test, is indicated (***$P < 0.001$, *$P < 0.01$).

ZMP, i.e. AICAR [16]. Preliminary experiments revealed that incubation of adipocytes with 500 μM AICAR increased the activity of partially purified AMPK in a time-dependent manner. AMPK activity following AICAR treatment reached a maximum by 30–60 min and remained elevated for approximately 120 min before returning to control levels (data not shown). Fig. 1A shows that AICAR activates cellular AMPK in a dose-dependent manner with the maximum stimulation (~3-fold measured in the presence of a saturating concentration of 5'-AMP) being achieved at a concentration of approximately 500μM.

3.2. *Effects of AICAR on lipolysis*

Preliminary experiments showed that neither basal or isoprenaline stimulated lipolysis could be inhibited when subsequently challenged with 500 μM AICAR for 1 h (data not shown). However, as shown in Fig. 2, preincubation of adipocytes with 500 μM AICAR followed by incubation with 10^{-9} to 10^{-5} M isoprenaline produced a shift to the right in the dose-response curve, with AICAR increasing the EC_{50} of isoprenaline from 5 ± 1 nM to 20 ± 9 nM. In addition, preincubation of adipocytes with 0–500 μM AICAR for 60 min followed by incubation with 10 nM isoprenaline produced a dose-dependent inhibition of isoprenaline-stimulated lipolysis (Fig. 1B).

3.3. *Effects of AICAR on lipogenesis and acetyl-coA carboxylase activity*

Incubation of rat adipocytes with AICAR led to a profound inhibition of lipogenesis in a dose-dependent manner (Fig. 1C). We also obtained a similar dose-dependent inhibition of [^{3}H]glucose incorporation into fatty acids when total lipids were saponified and the extracted fatty acids counted (data not shown). Based on our earlier observation of a dose-dependent activation of cellular AMPK by AICAR, we investigated whether the inhibitory effects of AICAR on lipogenesis were mediated through inhibition of ACC. Polyethylene glycol precipitates from AICAR-treated adipocytes were assayed for ACC activity in the presence of 10 mM citrate. As shown in Fig. 1D, AICAR caused a marked decrease in the V_{max} of ACC resulting in a 40% inhibition of activity with 500 μM AICAR.

To further investigate the mechanisms by which AICAR inhibits ACC activity, the ACC in PEG pellets from control and AICAR treated adipocytes was prepared and resuspended in buffer with or without the phosphatase inhibitors NaF and sodium pyrophosphate. The results in Fig. 3 show that the inhibitory effects of AICAR on ACC activity can be reversed in vitro if the enzyme is prepared and assayed under conditions where endogenous protein phosphatases are likely to be active [20,21]. In addition, the reactivation of ACC by endogenous phosphatases can be blocked by the phosphatase inhibitor okadaic acid (Fig. 3). Taken together, these results suggest that the inhibitory action of AICAR on ACC is most likely due to increased phosphorylation of the enzyme by AMPK.

4. Discussion

Phosphorylation of HSL in intact rat adipocytes occurs on two distinct sites termed the basal and regulatory sites although the physiological role of the basal site is not clear. Nevertheless, it has been proposed that by phosphorylating the basal site, AMPK plays an antilipolytic role in vivo [14]. Recently, we have identified several analogues of 5'-AMP that activate rat and human AMPKs in vitro [15]. Clearly, agents that can activate AMPK in isolated cells would be useful in determining the role of this protein kinase in lipolysis.

Our results show that AICAR markedly activates AMPK in adipocytes in a time and dose-dependent manner. Although we have not measured ZMP levels in cellular extracts, it is likely that the effect of AICAR on AMPK activity is mediated by a rise in the intracellular concentration of ZMP, since AICAR

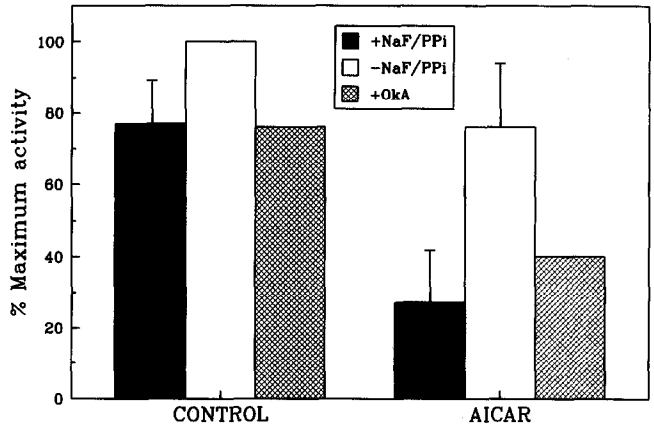

Fig. 3. Effect of AICAR on the phosphorylation state of ACC. Adipocytes were incubated with or without 500 μM AICAR for 60 min and ACC isolated and assayed in buffer containing 50 mM NaF/5 mM sodium pyrophosphate or 500 nM okadaic acid or buffer without phosphatase inhibitors. 100% activity is defined as the activity of ACC isolated from adipocytes in the absence of phosphatase inhibitors. The data points represent the mean of five separate experiments (except for okadaic acid where 2 experiments were performed), with the standard deviations from the mean indicated by the vertical bars.

also increases ZMP in other cell types [16,22]. The fact that the increased activity of AMPK survives partial purification suggests that the stimulation of AMPK by AICAR involves covalent modification of the kinase. This is supported by the observation that the increased activity of AMPK is detectable when the allosteric site is saturated (Fig. 1A) The most likely mechanism for the observed effects is an increased phosphorylation of AMPK, catalysed by the AMP-activated protein kinase kinase (AMPKK) in response to raised intracellular levels of the 5′-AMP analogue, ZMP. Recent work has shown that the phosphorylation and activation of AMPK by AMPKK is markedly stimulated by AMP. This effect appears to be mediated by the binding of 5′-AMP to AMPK [9] and so it is likely that ZMP can mimic this effect.

As anticipated, incubation of adipocytes with AICAR had no effect on the basal activity of HSL. However, the response of adipocytes to isoprenaline was markedly reduced if these cells were first preincubated with AICAR. The need for a preincubation step with AICAR is consistent with earlier in vitro data which shows that phosphorylation of HSL by AMPK prevents its subsequent activation [14].

AICAR also profoundly inhibits lipogenesis by increasing the phosphorylation of ACC, an enzyme known to be regulated by AMPK in cells and in vivo [8,23]. Evidence from other studies also shows that activation of AMPK leads to a reciprocal inactivation of its cellular targets, i.e. ACC and HMG-CoA reductase [7,9,24]. Additionally, a previous report has shown that AICAR profoundly inhibits both ACC and HMG-CoA reductase activities in primary rat hepatocytes, presumably by activating cellular AMPK [25].

We believe that our results provide the first cellular evidence to support the concept that AMPK plays a role in the physiological regulation of HSL. Further studies with AICAR and other cell-permeable activators of AMPK will investigate the mechanism(s) by which AMPK prevents activation of HSL. AMPK activators should also prove useful for studying the role of this kinase in the regulation of other cellular processes.

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