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Activation of glycogen phosphorylase and glycogenolysis in rat skeletal muscle by AICAR — an activator of AMP-activated protein kinase

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Abstract **We determined whether the cell permeable molecule AICAR, whose metabolite activates AMP-activated protein kinase (AMPK) in cells, affected glycogen metabolism in rat soleus muscle preparations in vitro. The basal and insulin-stimulated rates of radiochemical lactate formation, net lactate release and glycogen synthesis were determined. AICAR stimulated net lactate release (but not radiochemical lactate formation) only at a basal concentration of insulin. An increased rate of glycogenolysis was the likely cause of increased net lactate release as glycogen phosphorylase activity was significantly increased by AICAR. AICAR-stimulated net lactate release and phosphorylase activity were potently inhibited by insulin.**

Key words: Skeletal muscle; AMP-activated protein kinase; Glycogen phosphorylase; Insulin resistance; Glucose; Lactate

1. Introduction

Insulin is a potent stimulator of glucose transport and intracellular glucose disposal in skeletal muscle. Insulin regulates glucose disposal by controlling the recruitment of GLUT 4 glucose transporter protein to the cellular membrane and the activity of key enzymes such as hexokinase-II, glycogen synthase and phosphorylase. Recently, it has been suggested that in skeletal muscle glycogenolysis is more sensitive to insulin than is glucose transport and/or phosphorylation [1]. Glycogen phosphorylase, the flux-generating enzyme of glycogenolysis, is an important determinant of the rate of glycogenolysis. Acute regulation of glycogen phosphorylase activity consists of allosteric modulators and protein phosphorylation. However, the regulation of phosphorylase activity in skeletal muscle is not completely understood [2].

Several enzymes are potential substrates for AMP-activated protein kinase (AMPK), including glycogen synthase and phosphorylase kinase [3]. AMP-kinase is acknowledged as performing a major role in regulating lipid metabolism in adipose cells [4–6] and liver cells [7–9]. AICAR is a cell permeable molecule that is taken up readily by cells (probably by the adenosine transporter) and phosphorylated, by adenosine kinase [10], to ZMP. Thereafter ZMP may be metabolised to inosine monophosphate (IMP) or ZDP or ZTP [10]. AICAR is converted to ZMP which affects a number of metabolic processes in adipocytes [6] and hepatocytes [11]. Recently, evidence for the metabolic effects of AICAR being mediated via activation of AMPK has been reported from experiments with adipocytes [6,12] and hepatocytes [12]. An elevation of ZMP will mimic the effect of AMP which activates AMPK both by direct allosteric activation [13] and via promoting increased phosphorylation catalysed by a kinase kinase [14]. AICAR is taken up by skeletal muscle cells and metabolised to ZMP [15,16] but the effects of AICAR on glucose metabolism in muscle have not been reported.

As glycogen synthase and phosphorylase kinase are potential substrates for AMPK [7] we hypothesised that incubation of skeletal muscle preparations with AICAR would lead to alterations in the catalytic activities of these enzymes (via ZMP and possible ZMP activated AMPK) which would result in the inhibition of glycogen synthesis and activation of glycogen breakdown. We also investigated whether insulin had any effect on glycogen metabolism altered by AICAR.

2. Materials and methods

2.1. Animals

Male Wistar rats (130–140 g) were purchased from Harlan-Olac, Bicester, UK, and were kept in the Departmental animal house until experimentation. The animals were housed in groups of six under a controlled 12-h light–dark cycle (lights on from 08.00 to 20.00 h). Food (standard laboratory chow supplied by SDS, Whitmam, UK; digestible carbohydrate 52%, protein 16%, fat 2%, non-digestible residue 30% all by weight) and water were provided ad libitum, until 12 h before the animals were killed. Stripped soleus muscle was routinely prepared between 08.30 to 09.30 h.

2.2. Incubation procedures

Rats were killed by cervical dislocation, and soleus muscles were isolated and dissected longitudinally, rapidly weighed and tied at the resting length in situ on stainless steel clips, and placed in 25-ml Erlenmeyer flasks containing oxygenated (3 ml) Krebs-Ringer bicarbonate buffer (pH 7.4), with 5.5 mM glucose and 10 μU/ml insulin, as previously described [17,18]. Muscles were pre-incubated with shaking (100 rpm) for 40 min in sealed flasks at 37°C and then transferred to separate flasks containing Krebs-Ringer bicarbonate buffer (3 ml) containing 5.5 mM glucose (0.5 μCi [U-^{14}C]glucose/ml), 1% bovine serum albumin, and insulin at the concentration given in the section 3.1–3.8 (the details of the protocol have been given previously [17,18]. When phosphorylase activity was measured in the isolated incubated soleus muscle preparations [U-^{14}C]glucose was omitted from the medium. In addition, where the effects of AICAR were tested, 1 mM AICAR was added to both the pre-incubation and the incubation flasks (i.e. muscle incubated for 100 min with AICAR).

2.3. Measurement of glycolysis and glycogen synthesis

After 60 min of incubation at 37°C, muscles were removed from the incubation flasks and frozen in liquid nitrogen; the concentration of lactate in the medium was always determined. In certain cases the rate of incorporation of [U-^{14}C]glucose into glycogen, the rate of release of [^{14}C]lactate into the medium, and the activity of glycogen phosphor-

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Abbreviations: AMPK, AMP-activated protein kinase; AICAR, 5-amino-4-imidazolecarboxamide ribonucleoside; ZMP, 5-amino-4-imidazolecarboxamide ribonucleotide

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ylase were determined. ^{14}C-radiolabelled lactate release is a good indicator of the rate of glucose transport into isolated incubated skeletal muscle preparations [19]. Net lactate release is a term used to describe lactate that can arise from glucose transported into the cell and glucosyl units release from glycogen breakdown.

2.4. Glycogen phosphorylase assay

Frozen muscle tissue was crushed and homogenized (10 s) in 1 ml of 20 mM 6-phosphoglycerate and 200 μl of Solution A [60% (vol/vol) glycerol, 20 mM NaF and 1 mM EGTA; pH 7.5] by use of a polytron homogeniser [20]. The homogenate was centrifuged for 2 min at 13000×*g*. The supernatant was diluted 1:1 with buffer B (50 mM MES, 50 mM KF and 60 mM β-mercaptoethanol; pH 6.1). The diluted supernatant (0.06 ml) was mixed with 100 μl of the reaction mixture C (400 mM KF, 2 mg/ml glycogen, 27.88 mg/ml glucose-1-phosphate, 0.5 μCi/ml [U-^{14}C]glucose 1-phosphate, and various test metabolites — see section 3.2) and incubated for 1 h at 30°C. A portion of the mixture (0.08 ml) was removed and placed onto 20×20 mm 3M chromatography paper. The paper was dropped immediately into 66% (v/v)-ethanol (4°C). Three separate washes in ice-cold 66% (v/v) ethanol were preformed. The papers were removed and bathed in acetone for 2–3 min before being dried. Each paper was covered with scintillation fluid before being counted in a scintillation counter. A wash blank was preformed with Buffer A (0.06 ml). Results are expressed as μmoles of glucosyl units assimilated into glycogen per minute per gram of protein in the diluted supernatant (μmol/min per g protein). Protein content was measured by the Bio-Rad assay.

2.5. Statistical analysis

Student's *t* test was used for comparison of significance of differences between two sample means.

3. Results

3.1. Glycogen synthesis and glycolysis

AICAR did not alter the rate of glycogen synthesis in rat soleus muscle preparations at either a basal or a maximally-stimulating concentration of insulin (i.e. 10 and 1000 μUnits/ml; see Fig. 1). The effects of AICAR on basal and insulin-stimulated rates of net lactate release are given on Fig. 2. AICAR had no effect on glycolysis in the presence of a sub-maximal (100 μUnits/ml) or maximal concentration of insulin.

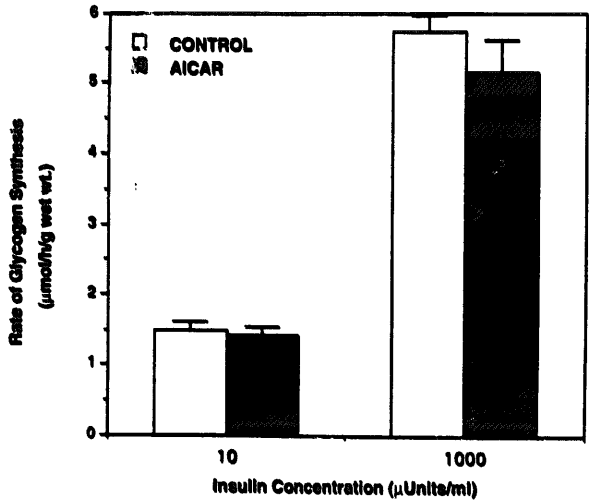

Fig. 1. Effect of 1 mM AICAR on basal and insulin-stimulated rates of glycogen synthesis in incubated soleus muscle preparations. AICAR was added to the pre-incubation and incubation media. The results are presented as the mean values ± S.E.M. for at least ten separate incubations.

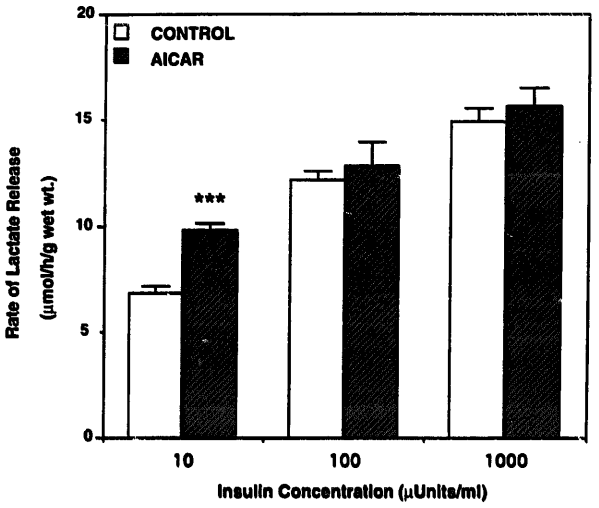

Fig. 2. Effect of 1 mM AICAR on basal and insulin-stimulated net lactate release in isolated incubated soleus muscle preparations. AICAR was added to the pre-incubation and incubation media. The results are presented as the mean values ± S.E.M. for at least ten separate incubations. Significant differences from control values are indicated by ***$P < 0.001$.

However, at a basal concentration of insulin there was a significant increase in the rate of lactate release (Fig. 2). The increased rate of lactate release may be the result of increased rate of glucose transport which would be indicated by an increase in the rate of ^{14}C-labelled lactate release. AICAR (1 mM) [4.26 ± 0.45 μmol/h per g wet wt. (10)] did not affect the basal rate of ^{14}C-labelled lactate release compared with control a value [3.98 ± 0.37 μmol/h per g wet wt. (10)].

3.2. Phosphorylase activity

The activity of glycogen phosphorylase was assayed in rat soleus muscle preparations incubated with a basal concentration of insulin (10 μUnits/ml) and in the absence or presence of AICAR (1 mM). AICAR significantly increased (55%) glycogen phosphorylase activity in isolated incubated soleus muscle preparations (Fig. 3a).

Effectors (AMP or AICAR or ZMP) that might have altered phosphorylase activity in vitro were added to the muscle homogenates prepared from soleus muscle preparations incubated with or without AICAR (Fig. 3b). AMP (4 mM), as previously reported [2], significantly increased the activity of glycogen phosphorylase in muscles incubated in the absence or presence of AICAR (Fig. 3b). Furthermore, AICAR present in the incubation medium caused a significantly additive increase in phosphorylase activity already stimulated with AMP (Fig. 3b). AICAR (4 mM) added to the glycogen phosphorylase reaction mixture did not affect phosphorylase activity in muscle preparations incubated in the absence or presence of AICAR (Fig. 3b). However, the phosphorylated form of AICAR, ZMP (4 mM), did significantly increase the phosphorylase activity by a similar magnitude as that seen with 4 mM AMP (Fig. 3b). The concentration-dependent effects of AMP and ZMP on the phosphorylase activity were measured in fresh (i.e. non-incubated) soleus muscle homogenates (Fig. 4). The percentage stimulation of phosphorylase activity was 49%, 100%, 146%, 177% and 162% at 0.075, 0.2, 0.5, 4 and 10 mM AMP, respectively (see Fig. 4). ZMP significantly stimu-

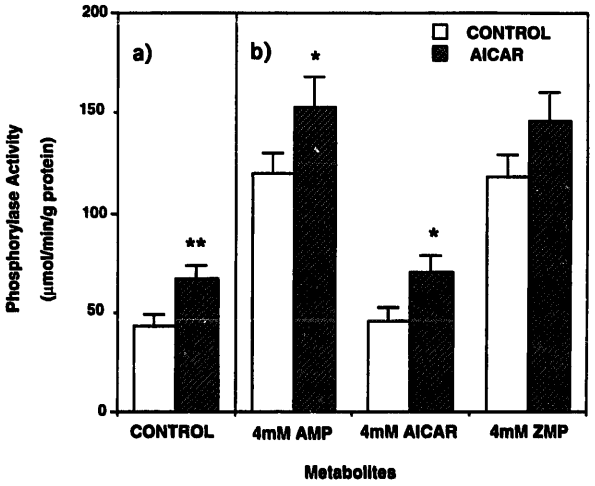

Fig. 3. Glycogen phosphorylase activity in homogenates prepared from solei incubated in the absence or presence of AICAR (a) and in homogenates incubated with AMP, AICAR or ZMP (all at 4 mM) (b). Glycogen phosphorylase activity was assayed as described in section 2.4. Muscles were incubated with or without AICAR before measurement of phosphorylase activity (Fig. 4a). AMP, AICAR or ZMP (all 4 mM) were added to muscle homogenates prepared from solei incubated with or without AICAR (b). The results are presented as the mean values ± S.E.M. for at least ten separate incubations. Significant difference from control values are indicated by $^{**}P < 0.01$ or $^{*}P < 0.05$.

lated phosphorylase activity at 0.2 mM (17%), 0.5 mM (71%) and 4 mM (174%).

3.3. *Effect of insulin*

The effect of insulin on AICAR-stimulated rates of lactate release and glycogen phosphorylase activity are given in Fig. 5. AICAR significantly stimulated both lactate release and phosphorylase activity (Fig. 5). Insulin, at a sub-maximal concentration of 100 μUnits/ml, deceased the AICAR-stimulated rate of lactate release by 89% and phosphorylase activity by 78% (Fig. 5).

4. Discussion

The aim of the present study was to determine the metabolic effects of AICAR on glycogen metabolism in an isolated incubated rat skeletal muscle preparation. AICAR had no effect on basal or insulin-stimulated glycogen synthesis (Fig. 1). However, the presence of AICAR (1 mM) in the pre-incubation and incubation medium caused a significant increase in the rate of net lactate release in soleus muscle preparations at a basal, but not at sub-maximal or maximal concentrations of insulin (Fig. 2). The basal rate of ^{14}C-labelled lactate release was not affected by AICAR (see section 3.1) which indicated that the rate of glucose transport into the muscle cells was unaffected. This suggested that AICAR stimulated the rate of glycogenolysis, which was confirmed by the fact that AICAR significantly increased glycogen phosphorylase activity in isolated incubated rat soleus muscle preparations (Fig. 3a and b). AICAR did not activate glycogen phosphorylase activity in skeletal muscle homogenates (Fig. 3b) which demonstrates the need for AICAR to be converted to an active metabolite (i.e. ZMP).

Glycogen phosphorylase exists in two forms, *a* and *b*, which are interconvertible via phosphorylation (by phosphorylase kinase) and dephosphorylation (by a phosphatase). In resting muscle, most (probably 99%) of the phosphorylase is in the *b*-form [21]. Phosphorylase *b* can be activated by phosphate, AMP and IMP. In resting muscle the concentration of phosphate, AMP and IMP are such that the phosphorylase *b* form is not activated. Very large changes in the concentration of phosphate, AMP and IMP are required to activate phosphorylase *b*.

We can postulate about the mechanism(s) for AICAR-stimulated glycogenolyis in skeletal muscle. Two hypotheses can be forwarded. Firstly, the accumulation of ZMP and/or IMP in soleus muscle preparations in vitro leads to an allosteric activation of glycogen phosphorylase. Secondly, accumulation of ZMP binds to AMPK and this results in activation of this enzyme. AMPK can phosphorylate phosphorylase kinase in vitro [3], although whether this occurs in vivo to change catalytic activity has yet to be demonstrated. The activated phosphorylase kinase would provide a mechanism for activation of both glycogen phosphorylase and glycogenolysis.

The concentrations of ZMP and IMP necessary for half-maximal activation of phosphorylase *b* are about 0.6 mM (see Fig. 4) and 1–2 mM, respectively. These concentrations are equivalent to a muscle content of between 0.7 to 1.4 μmol/g wet wt. During infusion of 8 millimoles of AICAR to dogs over 40 min the peak concentration of AICAR in the plasma at the end of infusion is 0.336 mM [22,23]. ZMP content reached a maximum of 0.032 μmol/g wet wt. and IMP content is 0.05 μmol/g wet wt. Assuming a cytosol water content of 0.52 ml/g [24] and a linear concentration and time-dependent relationship for AICAR phosphorylation to ZMP and IMP (for both in vivo and in vitro) the highest estimated concentrations for these metabolites in soleus muscle cells are about 460 and 720 μM, respectively. Hepatocytes incubated with 0.5 mM AICAR have an intracellular concentration of ZMP of

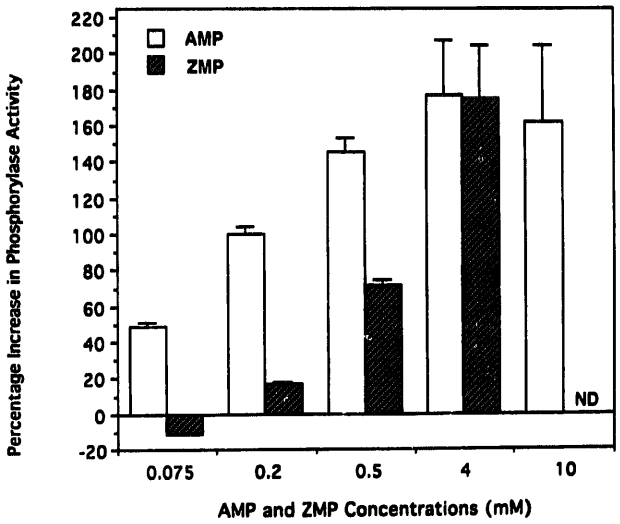

Fig. 4. Effects of AMP or ZMP on glycogen phosphorylase activity in muscle homogenates prepared from rat solei. Solei were rapidly isolated and immediately frozen in liquid nitrogen. Results are presented as the percentage increase in phosphorylase activity in the presence of metabolite compared with the activity in the absence of metabolite. N.D. means not determined. The results are given as mean values ± S.E.M. for at least 5 separate observations.

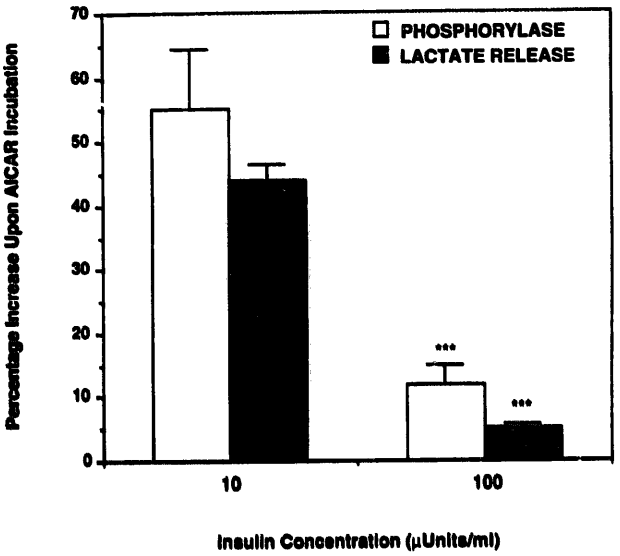

Fig. 5. Effects of insulin on the percentage increase of AICAR-stimulated glycogen phosphorylase activity and net lactate release in soleus muscle in vitro. Glycogen phosphorylase activity was assayed as described in section 2.4. Muscles were incubated with AICAR and 1 or 10 μUnits of insulin/ml before measurement of phosphorylase activity and net lactate release. The results are given as mean values ± S.E.M. for at least 8 separate observations. Significant differences from control values are indicated by $***P < 0.001$.

10 mM. This concentration could not occur in rat skeletal muscle because the activity of adenosine kinase, the key regulatory enzyme for adenosine (AICAR) phosphorylation, is 50-fold higher in rat liver [25]. Therefore, it would seem unlikely that upon AICAR metabolism the concentrations of ZMP and/or IMP are sufficiently high enough for them to act as direct allosteric activators of glycogen phosphorylase in muscle. Also, the glycogen phosphorylase assay involves a 130-fold dilution of the homogenate and, therefore, the allosteric effect of ZMP on phosphorylase would be lost. It is clear that in soleus muscle preparations incubated with AICAR a significant effect on phosphorylase does survive the homogenisation procedure, even with AMP (Fig. 3b). This must mean AICAR stimulated phosphorylase activity is by a different mechanism.

We suggest that ZMP (from AICAR) activates AMPK which phosphorylates, to activate, phosphorylase kinase which is the kinase responsible for activation of glycogen phosphorylase. AMPK activity, although low, is detectable in skeletal muscle [26]. The half-maximal effect of AMP on rat liver AMPK is between 4.4 and 29 μM (depending on the assay buffer ATP concentration) [20]. ZMP is known to mimic the effects of AMP by allosterically activating rat and human liver AMPK and rat adipocyte AMPK [8]. ZMP is both an allosteric activator of AMPK and, via AMP-activated protein kinase kinase, promotes the re-activation of dephosphorylated AMPK [14]. The K_a for allosteric activation of AMPK by ZMP is 81 μM (rat liver) or 103 μM (rat adipocyte). If the K_a for activation of skeletal muscle AMPK by ZMP is comparable with rat adipocyte or liver enzymes then we believe enough AICAR was metabolised to ZMP (estimated to be 460 μM) to cause activation of AMPK in the isolated soleus muscle preparations. Clearly, future studies should establish the magnitude and degree of allosteric activation of skeletal muscle AMPK by ZMP. The lack of studies on measurement of AMPK activity in skeletal muscle cells suggests there are problems with the assay of this enzyme in this tissue. Nevertheless, future studies will be needed to determine the effect of AICAR on AMPK activity and the phosphorylation status and activity of phosphorylase kinase in skeletal muscle.

Finally, the present study has shown that the AICAR-stimulated increase in phosphorylase activity or net lactate release is potently inhibited by insulin (Fig. 5). The concentration of insulin required for half-maximal activation of processes like glucose transport, glycogen synthesis or glucose oxidation in skeletal muscle is about 100 μUnits/ml [15,17]. The AICAR-stimulated rate of net lactate release and phosphorylase activity is inhibited by almost 90% at 100 μUnits of insulin/ml (Fig. 5). This finding agrees with results from an in vivo rat study that shows that glycogenolysis in skeletal muscle is more sensitive to insulin than is glucose transport and phosphorylation [1]. Interestingly, if AICAR does activate phosphorylase kinase our results suggests that phosphorylase kinase does not phosphorylate to inactivate glycogen synthase (Fig. 1), as has been previously suggested [27].

In conclusion we have shown that AICAR activates glycogenolysis and stimulated glycogen phosphorylase activity in skeletal muscle by ZMP. These effects are possibly caused by a mechanism involving activation of AMPK, rather than direct allosteric activation of phosphorylase by ZMP. During contraction or electrical stimulation or anoxia in skeletal muscle there is a significant increase in the concentration of free AMP [28]. We speculate that a rise in AMP during contraction is a novel mechanism to activate glycogenolysis via activation of AMPK.

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