AICAR acutely stimulates skeletal muscle 2-deoxyglucose uptake in healthy men.

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Running Title: AICAR stimulation of human glucose uptake

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Abstract

**Objective:** Activation of AMP-activated protein kinase (AMPK) in rodent muscle, by exercise, metformin, 5-aminimidazole-4-carboxamide 1-β-D-ribofuranoside (AICAR) and adiponectin, increases glucose uptake. The aim of the study was to determine whether AICAR stimulates muscle glucose uptake in humans.

**Research Design and Methods:** We studied twenty nine healthy men (26 ± 8 y, BMI 25 ± 4 kg.m^{-2}; mean ± SD). Rates of muscle 2-deoxyglucose (2DG) uptake were determined by measuring accumulation of total muscle 2DG (2DG and 2DG-6-phosphate, 2DG6P) during a primed, continuous 2DG infusion. The effects of AICAR and exercise on muscle AMPK activity/phosphorylation and 2DG uptake were determined. Whole body insulin sensitivity was compared before and during AICAR with the euglycaemic, hyperinsulinaemic clamp.

**Results:** Muscle 2DG uptake was linear over 9 h ($R^2 = 0.89 ± 0.08$). After 3 h, 2DG uptake increased 2.1 ± 0.8 and 4.7 ± 1.7 fold in response to AICAR or bicycle exercise respectively. AMPK-$\alpha_1$ and $\alpha_2$ activity, or AMPK phosphorylation, was unchanged after 20 min or 3 h of AICAR but AMPK phosphorylation significantly increased immediately and 3 h after bicycle exercise. AICAR significantly increased phosphorylation of extracellular-signal regulated kinase (ERK1/2), but phosphorylation of $\beta$–acetylCoA carboxylase ($\beta$–ACC), glycogen synthase (GS), protein kinase B (PKB) or insulin receptor substrate (IRS1) level was unchanged. Mean whole body glucose disposal increased by 7 % with AICAR from 9.3 ± 0.6 to 10 ± 0.6 mg.kg^{-1}.min^{-1} (P<0.05).

**Conclusion:** In healthy people, AICAR acutely stimulates muscle 2DG uptake with a minor effect on whole body glucose disposal.

**Keywords:** skeletal muscle, AMPK, glucose uptake, AICAR, ERK1/2.
Skeletal muscle is the major site of whole body glucose disposal during insulin stimulation or after exercise. Muscle glucose transport is mediated through the glucose transporter proteins GLUT1 and GLUT4 (1), with most of the effects of exercise or insulin on glucose uptake due to GLUT4 translocation. These two stimuli utilise distinct signalling pathways and are synergistic (2,3).

In type 2 diabetes (T2DM), insulin resistance results in impaired insulin-mediated skeletal muscle glucose transport (4), due to defects in GLUT4 trafficking rather than changes in protein expression (5,6). In contrast, exercise mediated glucose uptake is preserved (7). Hence exercise may be used to prevent and treat T2DM (8), implicating the signalling pathways that couple exercise with glucose transport as potential therapeutic targets.

AMPK is activated during exercise in rodent muscle (9-11) and is proposed to mediate the effects of exercise on muscle glucose uptake. AMPK may be pharmacologically activated by AICAR, which undergoes intracellular metabolism to ZMP which mimics the AMP effects on AMPK signaling (12). Perfusion of rat hindlimb muscle with AICAR caused accumulation of ZMP, activating AMPK and increasing glucose uptake (13). In rat muscle AICAR increases 2DG uptake due to an effect on transport rather than metabolism (14). Muscle AMPK is also activated by metformin (15), thiazolidinediones (16) and adiponectin (17), resulting in increased glucose uptake. In transgenic mice AMPK is necessary for the effect of AICAR on muscle glucose uptake. Knocking out AMPK-α2 (18), expressing a dominant negative AMPK mutant (19) or knocking out the upstream kinase, LKB1 (20), led to an abolition of the stimulatory effect of AICAR on muscle glucose uptake. However, the effects of contraction on glucose uptake was more variable, with an almost complete abolition of the effect in the LKB1-deficient mice (20), no reduction in the α2 knockouts (18), and a partial reduction in the mice expressing the dominant negative mutant (19).

In ex vivo human muscle, AICAR stimulates AMPK activity and glucose uptake two fold (21). Furthermore, in patients with T2DM, 4-10 weeks of metformin significantly increased muscle AMPK activity, glycogen concentration and whole body glucose disposal (22). However, 6 months of rosiglitazone, but not metformin, increased muscle glucose uptake during euglycaemic-hyperinsulinaemia and one-legged exercise (23). Although not measured, this suggests that muscle AMPK activity may not always correlate with glucose uptake.

AICAR is not a specific activator of AMPK. In rodent muscle and L6 myotubes AICAR activates the ERK (extracellular signal-regulated kinase)/atypical protein kinase C (aPKC) pathway resulting in increased glucose uptake (24). Similarly, metformin also acts through this accessory pathway, increasing aPKC basal expression, and its response to insulin stimulation, promoting skeletal muscle glucose uptake (25).

The in vivo effect of AICAR on whole body or skeletal muscle glucose uptake has not been examined in people. We determined whether AICAR would acutely increase muscle AMPK activity and glucose uptake or whole body insulin
sensitivity. 2-deoxyglucose (2DG) uptake was determined as a surrogate marker of muscle glucose uptake.

**Research Design and Methods**

**Subject characteristics**

Twenty nine healthy men (26 ± 8 y; BMI 25 ± 4 kg.m\(^{-2}\); mean ± SD) participated in the study. The subjects were habitually active at a recreational level. They adhered to their usual diet and refrained from strenuous physical activity for two days prior to the study. The study protocol was approved by the Tayside Ethics Committee and was carried out according to the Helsinki Declaration, with subjects giving their informed consent.

**Experimental protocol**

The subjects attended the laboratory having fasted from 2000 h on the previous evening. A forearm vein of each arm was cannulated at the antecubital fossae for infusion of the 2DG (Sigma, Poole, UK), and/or AICAR (Toronto Research Chemicals, Ontario), insulin (Actrapid, NovoNordisk, Denmark), 20 % dextrose (MacoPharma, UK) and for blood sampling. 2DG was given as a primed, constant infusion (priming dose 10 mg.kg\(^{-1}\); infusion rate of 6 mg.kg\(^{-1}.h^{-1}\)), AICAR at a rate of 10 mg.kg\(^{-1}.h^{-1}\), insulin at 40 mU.m\(^{-2}.min^{-1}\) and a variable 20 % dextrose infusion to maintain plasma glucose at 5 mmol.l\(^{-1}\). Venous blood samples were taken to determine plasma 2DG, AICAR, glucose and insulin concentrations. Quadriceps muscle biopsies were taken under 1% lignocaine anaesthesia using the conchotome technique (26). All biopsies were taken through separate incisions, made from distal to proximal areas of the quadriceps.

**Protocol 1: Accumulation of 2DG in skeletal muscle**

To determine whether there was a linear increase in the muscle concentration of 2DG and 2DG6P under basal conditions, four volunteers (age 25 ± 2 y; BMI 23 ± 1 kg.m\(^{-2}\)) were given an infusion of 2DG for 9 h, with biopsies taken after 3, 6 and 9 h and total 2DG accumulation determined. No basal muscle biopsy was required as 2DG is not naturally occurring.

**Protocol 2: Effect of AICAR on skeletal muscle 2DG uptake and AMPK activity**

To determine whether AICAR increases skeletal muscle glucose uptake and AMPK activity, six volunteers (age 23 ± 3 y; BMI 25 ± 2 kg.m\(^{-2}\)) were given an infusion of 2DG over 6 h, with AICAR infusion starting after 3 h. Muscle biopsies were taken at 0, 3 and 6 h, with total 2DG accumulation, AMPK (total \(\alpha\) isoform) activity and signalling changes measured.

**Protocol 3: Effect of exercise on skeletal muscle 2DG uptake and AMPK activity**

To confirm that, using our methods, we can detect increases in muscle 2DG uptake or AMPK activity or phosphorylation, eleven men (age 29 ± 3; BMI 26 ± 1 kg.m\(^{-2}\)) undertook 1 h cycling at 70 % of maximum heart rate, which has been shown to increase glucose uptake and AMPK phosphorylation (27). In 4 subjects, 2DG was infused continuously and muscle biopsies were taken after 3 h (but immediately before exercise) and 3 h post exercise, and total muscle 2DG uptake determined. In 7 subjects, muscle biopsies were taken at rest, immediately after and 3 h after exercise and AMPK (Thr\(^{172}\)) phosphorylation measured.

**Protocol 4: Time course of AMPK activity**

To determine whether AICAR transiently activated AMPK, four volunteers (age 27 ± 2 y; BMI 25 ± 1 kg.m\(^{-2}\)) were given a 3
h AICAR infusion. Muscle biopsies were taken at 0, 20 min and 3 h and AMPK (α₁ and α₂) activity and signalling changes measured.

**Protocol 5: Hyperinsulinaemic, euglycaemic clamp**

To determine whether AICAR increased whole body glucose uptake, 20 % glucose was infused at a variable rate to maintain plasma glucose at 5.0 mmol/l under conditions of hyperinsulinaemia (40 mU.m⁻².min⁻¹) (28) for 3 h under basal conditions and for 3 h during an AICAR infusion. The amount of glucose metabolised (M value) was calculated, relative to total body weight.

**Analytical methods**

**Materials and antibodies**

Except where otherwise stated all chemicals were of the highest quality available from Sigma (Poole, UK). Antibodies to phospho-specific and total PKB and GSK3 were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The antibody to IRS1 (raised against 14 C-terminal amino acids) was purchased from Upstate Biotechnology (Lake Placid, NY, USA) and β-Actin was purchased from Sigma (Poole, UK). Phospho-specific ERK1/2 antibody was from Cell Signaling Technology Inc (Beverly, MA, USA) and phospho-specific GS was raised by the Division of Signal Transduction Therapy (DSTT) against a phosphopeptide based around site 3 of GS.

**Plasma**

Plasma was separated from whole blood by centrifugation (300 × g) immediately after collection, and the plasma frozen until further analysis. Plasma glucose and lactate concentrations were measured with a YSI Stat2300 (Yellow Spring Instruments, Yellow Spring, OH). Plasma insulin (Invitrogen, Paisley, UK), cortisol (Cambridge Bioscience, UK) and glucagon (CosmoBio, Japan) were determined using commercially available ELISA kits. AICAR was measured in perchloric acid extracts of plasma samples by capillary electrophoresis (20mM sodium phosphate buffer pH 6.4) with reference to a standard curve of AICAR spiked into untreated plasma. Plasma 2DG was quantified by gas chromatography-mass spectrometry (MD800, ThermoFinnigan, Hemel Hempstead, UK). Briefly, to a 200 µl aliquot of plasma, 10 µl of internal standard (fluorodeoxyglucose (FDG)) was added. The samples were passed through Dowex-CF resin and eluted with 3 ml of distilled water. The elutants were dried down and derivatized as the oxime/triethylsilyl derivative (29). The concentrations were calculated with reference to a standard curve of 2DG spiked into untreated plasma and extracted as above.

**Muscle**

2 deoxyglucose (2DG) and 2 deoxyglucose-6-phosphate (2DG6P)

Frozen muscle (30-40 mg) was ground under liquid nitrogen, and the frozen powder transferred to 70 % ethanol. The sample was vortex mixed then centrifuged at 5000 g for 10 min. From this sample, the pellet containing the protein and nucleic acids was retained for glycogen analysis and the supernatant used for 2DG/2DG6P analysis. The supernatant was dried down under nitrogen and reconstituted in ddw (pH 9). The sample was then split into two equal fractions: The first fraction had 10 µl FDG added for determination of free 2DG concentration. The second fraction had 20 µl 100 mM MgCl₂ and 1 unit of alkaline phosphatase (Promega, Madison, WI) added, the
sample was vortex-mixed and heated at 37°C for 50 min, 10 µl FDG was then added for determination of total 2DG and both fractions dried under nitrogen. The samples were then derivatized as above and concentrations determined with reference to a standard curve of known 2DG concentration spiked into untreated muscle and extracted as above. The concentration of 2DGP was calculated as the difference between total 2DG concentration and free 2DG concentration.

**Muscle metabolites and glycogen**

Muscle was extracted with PCA and subjected to capillary electrophoresis to determine intracellular nucleotide and ZMP concentrations as previously described (30). The metabolite concentrations were determined as a ratio relative to ATP in the basal muscle sample and then normalized to known ATP concentrations (31). The protein pellet, obtained after extraction of 2DG, was hydrolysed in 2N HCl at 100°C for 2 h, neutralized and the resulting free glucosyl units were assayed spectrophotometrically using a glucose oxidase/horseradish peroxidase assay (32).

**AMPK α-isoform specific activity**

Muscle lysates were prepared by homogenization of muscle tissue (1:20, wt/vol) in a buffer containing 50 mM HEPES (pH 7.5), 150 mM NaCl, 20 mM Na-pyrophosphate, 20 mM β-glycerophosphate, 10 mM NaF, 2 mM Na-orthovanadate, 2 mM EDTA, 1% Nonidet P-40, 10% glycerol, 2 mM PMSF, 1 mM MgCl₂, 1 mM CaCl₂, 10 µg/ml leupeptin, 10 µg/ml aprotinin, and 3 mM benzamidine. Lysates were obtained from the supernatant fraction after a 10 min centrifugation at 13000 g, and pre-cleared for 1 h at 4 °C with Protein G–Sepharose in PBS (50%, v/v) to remove contaminating antibodies present due to the variable amount of blood in the samples. 30–40 µg protein from the muscle lysates were separated on Novex SDS/4–12% polyacrylamide gels. Following transfer to nitrocellulose, blots were blocked with 5% (w/v) non-fat milk in TBST [Tris-buffered saline containing 0.1% (v/v) Tween 20] for 1 h, and incubated with primary antibodies at 4 °C overnight before incubation for 1 h at room temperature with the secondary antibody and development using an ECL (enhanced chemiluminescence) kit (Amersham Biosciences, Inc.).

For the muscle samples from the AICAR and 2DG infusion study, antibodies to PKB (Ser₃₀⁸, 1:1,000) and GSK3 (Ser ⁹/²¹, 1:2,000) were used. After phospho-
specific analysis, membranes were stripped with 1X Western Re-Probe (Oncogene Research Products, San Diego, CA) and incubated with: PKB (1:1,000) and GSK3 (1:2,000). Secondary antibodies for anti-rabbit (1:25,000) were from Pierce Biotech (Rockford, IL). Results were expressed relative to the total protein. For the muscle samples from the AMPK time course study, antibodies to ERK1/2 (P-Thr^{202}/Tyr^{204}, 1:1,000), GS (phosphosite 3, 1:1,000), PKB(P-Ser^{473}, 1:1,000), and IRS1(total, 1:1,000) were used. Results were expressed relative to β-actin (C-terminal fragment). Protein bands were scanned and quantified by densitometry using AIDA Image Analyzer software.

**Calculation of glucose uptake**
Rates of muscle 2DG uptake were calculated by determining the increase in the concentration of 2DG and 2DG6P between consecutive muscle biopsies over each time period, assuming this to be representative of all hexose uptake:

\[
2DGU = \frac{([DG + DG6P]_2 - [DG + DG6P]_1)}{t}
\]

Where 2DGU = deoxyglucose uptake, DG = concentration of 2-deoxyglucose, DG6P = concentration of 2-deoxyglucose 6 phosphate, t = time and the subscripted numbers 1 and 2 denote the first and second muscle biopsy samples in which the DG/DG6P concentrations are determined.

**Data and statistical analysis**
Data are expressed as means ± standard errors. Results were compared using a paired t-test or with a repeated measures ANOVA with Bonferroni post test (where there were 3 or more data sets). The null hypothesis was rejected at the 5% level (\(P < 0.05\)).

**Results**

**Accumulation of 2DG under basal conditions**
Plasma 2DG concentrations were at steady state throughout the study (data not shown). 2DG muscle uptake increased in a linear fashion in all 4 individuals (mean \(R^2 = 0.88 ± 0.09\)) (Figure 1).

**Glycogen content**
There was a non significant fall in muscle glycogen during the study from a 3 h concentration of 19 ± 1.9 to 14 ± 1.5 mmol. glucosyl units.kg\(^{-1}\) wet weight of muscle after 9 h.

**Plasma lactate and hormone concentrations**
Plasma lactate, cortisol and glucagon remained constant over the course of the study (1.9 ± 0.3 mmol.l\(^{-1}\), 7.8 ± 1.0 µg.dl\(^{-1}\) and 28.4 ± 8.4 pg.ml\(^{-1}\) respectively, grand means).

**Stimulation of muscle 2DG uptake during AICAR infusion and exercise**

**Plasma**

**2DG and glucose concentrations**
During the AICAR infusion, neither plasma 2DG nor plasma glucose (Table 1) concentrations were significantly different. The ratio of plasma 2DG:glucose was unchanged with AICAR. During exercise the ratio of plasma 2DG:glucose decreases non significantly immediately post exercise (Figure 2).

**Insulin and lactate concentrations**
Plasma insulin (Table 1) and lactate remained constant before and during the AICAR infusion (10.7 ± 2.2 mU.l\(^{-1}\) and 1.7 ± 0.5 mmol.l\(^{-1}\) respectively). During
exercise the plasma lactate concentration increased from \(1.7 \pm 0.7\) to \(3.6 \pm 1.5\) mmol.l\(^{-1}\) immediately after exercise and remained elevated during recovery \(2.7 \pm 1.7\) mmol.l\(^{-1}\).

**Muscle**

**2DG uptake**

There was a 2.1 fold increase in 2DG uptake after the 3 h AICAR infusion. In response to exercise, there was a 4.7 fold increase in 2DG uptake (Figure 3).

**Glycogen content**

There was a non significant fall in muscle glycogen concentration during the AICAR infusion study; basal: \(26 \pm 3.7\) 3 h: \(21 \pm 3.7\) 6 h: \(17 \pm 2.4\) mmol. glucosyl units.kg\(^{-1}\) wet weight.

**AMPK activity and phosphorylation of Acetyl CoA-Carboxylase (ACC)**

There was no significant change in total AMPK \(\alpha\)-isoform activity or in the phosphorylation of ACC, a downstream target of AMPK, in response to AICAR (Figure 4A). Phosphorylation of AMPK increased immediately after cycling exercise (474 \%; \(P<0.01\)) and remained elevated (162\%; \(P<0.05\)) 3 h post exercise (Figure 4C).

**Western analysis of signalling proteins** (data not shown)

In response to AICAR, PKB and GSK3 phosphorylation was unchanged.

**Time course of AMPK activity**

**Plasma**

**Glucose and hormone concentrations**

Glucose, insulin and glucagon concentrations were stable throughout the AICAR infusion at \(4.95 \pm 0.58\) mmol.l\(^{-1}\), \(6.5 \pm 2.2\) mU.l\(^{-1}\) and \(22.5 \pm 5.7\) pg.ml\(^{-1}\) respectively.

**AICAR concentration**

Venus plasma AICAR concentration remained constant throughout at \(0.18 \pm 0.03\) mM (Table 1).

**Muscle**

**AMPK activity and phosphorylation of AMPK and ACC**

AMPK \(\alpha_1\) and \(\alpha_2\) activity was unchanged after 20 min or 3 h of AICAR. Phosphorylation of AMPK or ACC also remained unchanged (Figure 4B/C).

**Western analysis of signalling proteins**

Phosphorylation of ERK1/2 increased after 20 min and was significant after 3 h of AICAR infusion (Figure 5). Phosphorylation of GS, PKB, and total IRS1 levels (Figure 5) were unchanged.

**Metabolites**

There was no change in the ratio of AMP, ADP and ATP during 3 h of AICAR infusion; by 3 h the ZMP concentration was 77\% of the AMP concentration, being undetectable at 0 or 20 min (Table 2).

**Effect of AICAR on whole body glucose uptake**

The M value increased in all 4 subjects by a mean of 7 \% from \(9.3 \pm 0.6\) to \(10 \pm 0.6\) mg.kg.min\(^{-1}\) (\(P<0.05\)). The plasma insulin values rose from a baseline value of \(9 \pm 1.7\) to \(159 \pm 11.4\) mU.l\(^{-1}\) after 180 min of insulin to \(171 \pm 10.7\) mU.l\(^{-1}\) after 180 min of insulin and AICAR (\(P<0.001\) baseline v 180 and 360 min). In contrast, plasma cortisol fell from a baseline value of \(7.5 \pm 0.4\) to \(5.8 \pm 1.1\) µg.dl\(^{-1}\) after 180 min of insulin to \(4.9 \pm 0.9\) µg.dl\(^{-1}\) after 180 min of insulin and AICAR (\(P<0.05\) baseline v 360 min). There was no change in plasma glucagon concentration (\(21.2 \pm 3.2\) pg.ml\(^{-1}\), grand mean).
Discussion
In healthy young men an acute AICAR infusion is associated with: (i) a two-fold increase in skeletal muscle glucose uptake after 3 h; (ii) no significant change in AMPK α₁ and α₂ activity; (iii) increased ERK1/2 phosphorylation; (iv) no change in phosphorylation of PKB, GS or total IRS1, and (v) a small change in whole body glucose disposal.

The *in vivo* effect of AICAR on skeletal muscle glucose uptake or on AMPK activation has not previously been examined in humans. We determined a surrogate measure of glucose uptake, 2DG, a glucose analogue that closely resembles glucose in the characteristics of its transport but is metabolized only to the 6-phosphate derivative. Thus 2DG6P is effectively trapped within skeletal muscle where its concentration (and that of 2DG) can be determined as a function of glucose uptake, assuming 2DG uptake to be representative of all hexose uptake. 2DG, as *¹⁴*C-2DG, *³*H-2DG (4) or *¹⁸*F-2DG (for PET) (23) is routinely used to determine changes in tissue glucose transport.

We believe that 2DG is a robust surrogate measure of glucose uptake and the rise in total muscle 2DG during any intervention is not due to methodological reasons (ie delayed equilibration of the plasma 2DG with the intracellular space). Post-absorptively, we demonstrate a linear accumulation of total muscle 2DG during a 9 h 2-DG infusion \( R^2=0.89 \pm 0.08 \). Secondly, exercise, a known stimulus of skeletal muscle glucose uptake significantly and consistently increased muscle 2DG uptake. 2DG is more likely to inhibit, rather than stimulate, glucose uptake via allosteric inhibition of hexokinase by 2DG6P (34), although 2DG6P is a very poor inhibitor (35). The linear accumulation of 2DG suggests that 2DG is neither stimulating (through a ‘mass effect’) nor inhibiting (through hexokinase inhibition) its own uptake over 9 h.

We observed a 2.1 fold stimulation of glucose uptake with AICAR, consistent with the results of animal studies (13,36). Incubation of human skeletal muscle strips with 1-2mM AICAR increased glucose transport 2.6-fold (21). The magnitude of the stimulation of 2DG uptake observed with AICAR was less than half that seen with acute exercise, a known stimulator of glucose uptake, and may explain the small effect of AICAR on whole body glucose disposal. Alternatively, the small whole body effect may be due to the increased muscle glucose uptake being counteracted by increased hepatic glucose output (37).

The stimulation of 2DG uptake following AICAR infusion for 3h is likely to be insulin independent. Insulin concentrations were at post-absorptive values throughout and components of the insulin regulated PI 3-kinase-dependent signaling pathway (PKB and GSK3) unaltered 3h post-AICAR. Unlike the results from animal studies or *ex vivo* human incubations, we saw no significant increase of AMPK activity after 20 min or 3 h of AICAR. This lack of stimulation of muscle AMPK activity with AICAR was surprising, in view of the evidence from studies in transgenic mice demonstrating that AMPK is essential for AICAR-induced glucose uptake (18,19,20). The lack of activation of AMPK at 3 h might reflect a transient activation of AMPK, similar to that observed in rat skeletal muscle in response to muscle contraction, with persistence of glucose uptake despite the rapidly decreasing AMPK activity.
In rat hepatocytes and adipocytes AMPK activation by AICAR peaks at 20 min and then rapidly falls (12). However, in additional time course studies we were unable to detect any change in AMPK activity or phosphorylation after 20 min of AICAR infusion, therefore transient activation was considered unlikely. Furthermore, phosphorylation of ACC, a downstream target of AMPK, did not change.

This failure to detect any rise in AMPK activity or phosphorylation was not due to our techniques, as immediately post-exercise we observed an ~5-fold increase in AMPK phosphorylation, which remained elevated at 3h. AMPK phosphorylation closely mirrors its activity, suggesting that our methodology would allow us to detect changes in kinase activity in response to AICAR.

There are several possible explanations why AMPK activation was not observed in parallel with the stimulation of glucose uptake after an AICAR infusion. It may be that in human skeletal muscle a small increase in AMPK activity (relative to exercise-induced AMPK activation) is sufficient to increase subsequent signalling pathways, which stimulate GLUT4 translocation and glucose uptake. However, AMPK activity normally increases 150-200% in response to treatments including AICAR, metformin or exercise (21,37,39,40). A lesser dose of AICAR (6 mg.kg^{-1}.h^{-1}) is clinically effective in preventing adverse cardiovascular outcomes in patients undergoing coronary artery bypass graft surgery (41). Therefore, this dose of AICAR has clinically and physiologically significant effects in cardiac muscle. Alternatively, AMPK activation in other tissues may regulate skeletal muscle glucose uptake. AMPK α2/-/- mice have reduced insulin-stimulated whole-body glucose utilization and muscle glycogen synthesis rates despite normal glucose transport in isolated muscles (42). This suggests an indirect action on muscle, perhaps via adipose tissue or through modulation of the sympathetic nervous system. A final possibility is that AICAR might be stimulating human muscle glucose uptake via an AMPK-independent action (43).

Among a range of signalling proteins, only phosphorylation of ERK1/2 (a member of the mitogen-activated protein kinase family) was significantly increased by AICAR; there were no changes in phosphorylation of PKB or GS or in total IRS1 expression. In cultured L6 cells, the stimulation of GLUT4 translocation and glucose uptake by AICAR has been shown to be mediated, at least partly, through sequential activation of MEK1/2 (the MAP kinase kinase), ERK1/2, PLD, phosphatidic acid and aPKCs (24). Furthermore, inhibition of any of the components of the ERK pathway (using a MEK1 inhibitor, a PLD inhibitor and expression of inactive ERK and PKC-ζ) abolished in parallel the AICAR–induced stimulation of glucose uptake. In our studies, the activation of ERK1/2 does not appear to be downstream of AMPK as we see no increase in AMPK activity, or phosphorylation, after 20 min whereas ERK1/2 phosphorylation has increased. Without the use of specific kinase inhibitors in human in vivo studies, it is not possible to definitively conclude that ERK1/2 mediates the stimulation of human skeletal muscle glucose uptake by AICAR. However, increased ERK 1/2 phosphorylation, rather than activity of AMPK, is associated with the stimulation of 2DG uptake.
A decrease in muscle glycogen concentration (~ 50 %) stimulates basal glucose uptake in rat skeletal muscle (44,45). However, we see a non-significant (~20 %) fall in glycogen concentration during the AICAR infusion. A similar reduction in glycogen concentration was observed in subjects infused only with 2-DG, with no effect on muscle glucose uptake. This suggests that increased 2-DG uptake following AICAR is mediated by AICAR itself and not falling glycogen stimulating glucose uptake.

Finally, the AICAR infusion is well tolerated and associated with no/minimal side effects in ours or other studies (41,46).

In summary, we report the first observations that acute in vivo AICAR administration increases human skeletal muscle 2-deoxyglucose uptake and whole body glucose disposal. In skeletal muscle, this was associated with ERK1/2 activation but with no effect on AMPK.

Acknowledgements
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References


Table 1 Time course of plasma glucose, insulin and AICAR concentrations

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<th>Time (min)</th>
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<tr>
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<td>Glucose (mM)</td>
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<tr>
<td>Insulin (mU.l⁻¹)</td>
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<td>AICAR (mM)</td>
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Table 2  Skeletal muscle metabolites (n=4 for each time point). ATP and ADP expressed as mmol.kg\(^{-1}\) muscle dry weight, AMP and ZMP expressed as μmol.kg\(^{-1}\) muscle dry weight.

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<th>Time (min)</th>
<th>ATP</th>
<th>ADP</th>
<th>AMP</th>
<th>ZMP</th>
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<tr>
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<td>180</td>
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**Figure legends**

**Figure 1** Linear accumulation of 2DG during a 9 h infusion with skeletal muscle biopsies taken at 3 hourly intervals (3, 6 and 9h), (n=4). Values are means ± SD.

**Figure 2** Ratio of plasma 2DG concentrations to plasma glucose concentration during a 6 h 2-DG infusion with a final 3 h AICAR infusion (black triangle, n=6) or exercise (white circle, n=4). Values are means ± SD.

**Figure 3** Rates of skeletal muscle glucose uptake (µmol.kg⁻¹.min⁻¹) before and after a 3 h AICAR infusion (n=6). Values are means ± SEM.

**Figure 4** A: AMPK α₁ and α₂ activity and ACC phosphorylation at baseline, after 2-deoxyglucose and after 3 h AICAR infusion; NS (n=6). B: AMPK α₁ and α₂ activity and ACC phosphorylation at baseline (n=4), after 20 (n=4) and 180 (n=4) min AICAR infusion. C: Representative Western blots of AMPK phosphorylation (Thr¹⁷²) at baseline (n=4), after 20 (n=4) and 180 (n=4) min AICAR infusion and at rest, immediately post exercise and 180 min post exercise (n=7).

**Figure 5** Representative Western blots of phospho ERK 1/2, GS, S6, PKB and total IRS1 at baseline (n=4), after 20 min (n=4) and 180 min AICAR infusion (n=3).
Figure 1

![Graph showing the relationship between time (h) and total 2-deoxyglucose accumulation (μg·g⁻¹ muscle wet weight).]

<table>
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<tr>
<th>Time (h)</th>
<th>2DG conc (μg·g⁻¹ muscle wet weight)</th>
<th>2DG-6-P conc (μg·g⁻¹ muscle wet weight)</th>
<th>Total 2DG conc (μg·g⁻¹ muscle wet weight)</th>
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<td>6</td>
<td>49.8 ± 27.6</td>
<td>81.9 ± 36.4</td>
<td>131.7 ± 63.6</td>
</tr>
<tr>
<td>9</td>
<td>69.9 ± 34.1</td>
<td>101.0 ± 36.6</td>
<td>171.9 ± 68.3</td>
</tr>
</tbody>
</table>
Figure 2

![Graph showing the ratio of plasma 2DG concentration to plasma glucose concentration over time. The graph compares Basal, Exercise, and AICAR conditions.](image)

**Y-axis:** Ratio Plasma 2DG conc.: Plasma Glu conc.

**X-axis:** Time (min)

- **Basal** (solid square)
- **Exercise** (dashed circle)
- **AICAR** (dotted triangle)
Figure 3

![Bar chart showing Total Muscle 2-DG uptake (µmol.kg⁻¹.min⁻¹) for Basal and +AICAR conditions, and Basal and +Exercise conditions. The chart includes error bars and statistical significance markers (P<0.05, P<0.01, P<0.001).]

<table>
<thead>
<tr>
<th></th>
<th>Muscle 2DG conc (µmol.kg⁻¹.min⁻¹)</th>
<th>Muscle 2DG-6P conc (µmol.kg⁻¹.min⁻¹)</th>
<th>Muscle total 2DG conc (µmol.kg⁻¹.min⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal</td>
<td>2.6 ± 1.2</td>
<td>4.7 ± 5.0</td>
<td>7.3 ± 4.9</td>
</tr>
<tr>
<td>+AICAR</td>
<td>2.7 ± 1.9</td>
<td>12.6 ± 4.5</td>
<td>15.3 ± 4.3</td>
</tr>
<tr>
<td>Basal</td>
<td>1.8 ± 0.8</td>
<td>4.0 ± 1.3</td>
<td>5.8 ± 1.6</td>
</tr>
<tr>
<td>+Exercise</td>
<td>5.2 ± 2.8</td>
<td>22.1 ± 6.1</td>
<td>27.3 ± 5.4</td>
</tr>
</tbody>
</table>
Figure 4

A

AMPK α1 & 2 activity (fold change from basal)

Time (min)

0 180 360
(2-DG) (AICAR+2-DG)

AMPK Phosphorylation (A.U.)

0 180 360
(2-DG) (AICAR+2-DG)

P-ACC

Total ACC

B

AMPK α1 activity (fold change from basal)

Time AICAR infusion (min)

Basal 20 180

AMPK α2 activity (fold change from basal)

Time AICAR infusion (min)

Basal 20 180

ACC phosphorylation (A.U.)

Basal 20 180

C

AMPK Phosphorylation (A.U.)

Time AICAR infusion (min)

0 20 180

Rest 0 180 min

P-AMPK (Tβ2)

Total AMPK

P-AMPK (Tβ2)

Total β-Actin
Figure 5