AMP kinase activation and glut4 translocation in isolated cardiomyocytes

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Summary

Activation of AMP-activated protein kinase (AMPK) results in glucose transporter 4 (GLUT4) translocation from the cytosol to the cell membrane, and glucose uptake in the skeletal muscles. This increased activation of AMPK can be stimulated by a pharmacological agent, AICAR (5′-aminoimidazole-4-carboxamide ribonucleoside), which is converted intracellularly into ZMP (5′-aminoimidazole-4-carboxamide-ribonucleosidephosphate), an AMP analogue. We utilised AICAR and ZMP to study GLUT4 translocation and glucose uptake in isolated cardiomyocytes.

Adult ventricular cardiomyocytes were treated with AICAR or ZMP, and glucose uptake was measured via [3H]-2-deoxyglucose accumulation. PKB/Akt, AMPK and acetyl-CoA-carboxylase phosphorylation and GLUT4 translocation were detected by Western blotting or flow cytometry.

AICAR and ZMP promoted AMPK phosphorylation. Neither drug increased glucose uptake but on the contrary, inhibited basal glucose uptake, although GLUT4 translocation from the cytosol to the membrane occurred. Using flow cytometry to detect the exofacial loop of the GLUT4 protein, we showed ineffective insertion in the membrane under these conditions. Supplementing with nitric oxide improved insertion in the membrane but not glucose uptake.

We concluded that activation of AMPK via AICAR or ZMP was not sufficient to induce GLUT4-mediated glucose uptake in isolated cardiomyocytes. Nitric oxide plays a role in proper insertion of the protein in the membrane but not in glucose uptake.

Keywords: AMPK, AICAR, GLUT4, GLUT4 exofacial loop, cardiomyocytes

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GLUT4 vesicles respond to insulin in a marked and dramatic way, increasing GLUT4 levels in the membrane up to nine times that of basal levels. The protein is recycled via endocytosis in clathrin-coated vesicles.

It has, however, been recognised that translocation of GLUT4 to the membrane, and glucose uptake by the GLUT4 transporters are not always reconcilable. There is a marked discrepancy between glucose uptake and translocation of GLUT4. Although our understanding of the regulation of GLUT4 translocation from the intracellular to the membrane compartments has expanded rapidly over the past years, the exact mechanisms governing these events, especially at the site of fusion with the cell membrane, is not fully understood. Events elicited by stimulation with insulin have been the focus of intense research, whereas less is known of those elicited by AMPK activation. However, stimulation with activators of AMPK share some of the downstream signalling events of the insulin pathway. Propagation of these events from the membrane to the intracellular location after AMPK activation was demonstrated that in cardiomyocytes, activation of AMPK was not sufficient to affect glucose uptake and GLUT4 translocation into the cell membrane and therefore glucose uptake.

Methods

Materials

AICAR, ZMP, insulin and Triton X-100 were obtained from Sigma, type 2 collagenase was from Worthington, bovine serum albumin (BSA) fraction V, fatty acid-free from Roche Diagnostics and 2-deoxy-D-[3H]glucose from New England Nuclear. The ECL Western blotting detection reagents, anti-rabbit Ig, horseradish peroxidase-linked whole secondary antibody were from Amersham Biosciences, UK Ltd, GLUT4 (H-61): sc-7938 rabbit polyclonal antibody was from Santa Cruz Biototechnology Inc., phospho-PKB/Akt (Ser473), phospho-AMPK-α (Thr172) and Phospho-ACC antibodies were from Cell Signalling Technology, The anti-GLUT4 (exofacial loop) was purchased from Chemicon International (Temecula, California, USA), and Zenon Alexa Fluor 488 Rabbit IgG Labeling Kit was acquired from Molecular Probes (Eugene, Oregon, USA). Paraformaldehyde was purchased from Merek (Cape Town, RSA) and Dulbecco’s phosphate-buffered saline (PBS) from Gibco (Grand Island, New York, USA).

Animals

Male Wistar rats weighing between 250 and 300 g were used in all experiments. Animals were bred and kept in the AAALAC-accredited facility of this institution. The project was approved by the Ethics committee of the Faculty of Health Sciences, University of Stellenbosch and conformed to the Guide for the Care and Use of Laboratory Animals of the NIH (Publication No. 85-23, revised 1996). Animals were anaesthetised with sodium pentobarbital (160 mg/kg) before experimentation.

Preparation of cardiomyocytes

Rod-shaped ventricular cardiomyocytes were obtained by collagenase perfusion, essentially as described previously. Isolated cells were filtered through a nylon mesh (200 × 200 µm) and gently spun down (3 min, 100 rpm). The resulting pellet was resuspended in HEPES buffer (10 mM HEPES pH 7.4, 6 mM KCl, 1 mM NaHPO4, 0.2 mM NaH2PO4, 1.4 mM MgSO4, 128 mM NaCl, 5.5 mM glucose, 2 mM pyruvate, containing 1.25 mM CaCl2, 2% BSA (fraction V, fatty acid free) and the cells were allowed to recover for 60 min on a slowly rotating platform. Osmotic fragility coupled to trypan blue exclusion (TBE) and cell morphology were used as indices for assessment of cell viability. Viable cells varied between 70% and 80% and all cell isolates of less than 70% viability were discarded. At the end of experimentation, the viability of cell preparations was determined with propidium iodide.

Propidium iodide staining

Propidium iodide (PI) staining was used to assess cell membrane permeability. Nuclear staining by PI was assessed with flow cytometric (FACS) analysis. Cardiac myocytes were incubated with 10 µM PI for 15 min before analysis. Data are expressed as mean fluorescence intensity as a percentage of control. FACS analysis was done with a FACSCalibur using Cellquest 3.3 software (Becton Dickinson, San Jose, California, USA).

Detection of glucose uptake

Myocyte uptake of 2-deoxy-D-[3H] glucose (2DG) was measured as described previously. Cardiomyocytes (approximately 0.5 mg protein) were placed in a total volume of 750 µl assay medium containing (in mmol/l): KCl 6, Na2HPO4 1, NaH2PO4 0.2, MgSO4 1.4, NaCl 128, HEPES 10, CaCl2 1.25 plus 2% BSA (fraction V, fatty acid free) pH 7.4, 37°C, equilibrated with oxygen. Cells were equilibrated for 15 min in a shaking water bath (180 strokes/min) with or without phloretin (400 µM) for measurement of non-carrier-mediated glucose uptake. They were stimulated with or without insulin (15 min × 100 nM), AICAR (30 min × 1 mM) or ZMP (30 min × 1 mM) as indicated. Glucose uptake was initiated by the addition of 2-deoxy-D-[3H] glucose (1.5 Ci/ml; final 2-deoxy-D-glucose concentration 1.8 µM) and allowed to progress for 30 min before the reaction was stopped with phloretin (final concentration 400 µM). The cells were then centrifuged and the pellet was washed twice with HEPES buffer and dissolved in 1 ml 0.5 N NaOH. An aliquot of this solution was then used to assay protein concentration by the method of Lowry, and the rest was counted for radioactivity.

Preparation of lysates for Western blotting

Cardiomyocytes were removed after stimulation with insulin, AICAR or ZMP as described above and placed on ice. The cells were centrifuged and washed three times with ice-cold HEPES buffer without albumin and lysed in 100 µl of lysis buffer (25 mM HEPES, 50 mM β-glycerophosphate, 1 mM EGTA, 1% Triton X-100, 10 mM p-nitrophenyl phosphate, 1 mM NaVO3, 2.5 mM MgCl2, 10 µg/ml aprotinin, 10 µg/ml leupeptin, 1 mM phenylmethylsulphonyl fluoride and 1 mM 1,4-dithiothreitol, pH 7.4). The lysates were centrifuged at 15 000 × g for 10 min and the supernatants diluted with Laemmli sample buffer for SDS-PAGE. An aliquot of the supernatant was used for protein determination.
Western blotting

After boiling for five minutes, equal amounts of sample protein from the various fractions were separated on a 10% SDS-PAGE and transferred to Immobilon®-P membranes. Transfer and equal loading were confirmed using the reversible stain, Ponceau red. Non-specific binding sites were blocked with 5% fat-free milk powder in TRIS-buffered saline (TBS) for two hours at room temperature and then incubated with either the phospho-Akt (Ser473), GLUT4 (H-61) : sc-7938 or phospho-AMPK (Thr172) primary antibody [1:1000 dilution in TBS plus 0.1% Tween-20 (TBST)] for 16 hours at 4°C. After washing with TBST, the membranes were treated for one hour with 1:4000 dilution of anti-rabbit Ig, horseradish peroxidase-linked whole antibody and the bands were visualised with the ECL method.

Membrane fractionation for measurement of GLUT4 translocation

In order to determine GLUT4 translocation, cardiomyocytes were fractionated into a sarcolemmal membrane and a cytosolic compartment, essentially as described by Takeuchi et al.38 After stimulation with the various compounds, the cardiomyocytes were washed twice with ice-cold HEPES buffer without albumin and then sonicated on ice in TES buffer containing (in mM): Tris-HCl 20, pH 7.5, EDTA 2, EGTA 0.5, sucrose 330 PMSF 1 and 25 µg/ml leupeptin. The homogenate was centrifuged to remove particulate debris (1000 × g at 4°C for 10 min).

The supernatant was further fractionated by ultracentrifugation for 90 min at 40 000 × g at 4°C (Beckman, Ti50). The supernatant obtained was considered to represent the cytosolic fraction. The pellet was suspended in the aforementioned buffer minus sucrose but containing 1% (vol/vol) Triton X-100 plus 1% SDS and rotated at 4°C for 30 min. Afterwards the suspension was ultracentrifuged at 40 000 × g for one hour at 4°C (Beckman, Ti50), and the supernatant used as the membrane fraction.

Protein concentration was determined using the method of Bradford,37 samples were diluted in Laemmli sample buffer, boiled for 5 min and stored at −20°C. GLUT4 content and distribution were determined by Western blotting and suitable antibodies.

Detection of GLUT4 exofacial loop

Approximately 100 000 cardiomyocytes per sample were used and washed once with PBS. After that, the cells were stained with 1 µg GLUT4 antibody coupled to Alexa Fluor 488 according to the manufacturer’s instructions, and washed with PBS. A fixing step with 1 ml PBS containing 1% paraformaldehyde (PFA) for 15 min at room temperature followed. Samples were centrifuged at 4 × g for 5 min. Flow cytometric analysis was done with a FACS Calibur using Cellquest 3.3 software (Becton Dickinson, San Jose, California, USA). A forward-scatter against side-scatter plot was used to distinguish the cells from the debris, as described previously.39 The fluorescence signal of the labelled antibody bound to GLUT4 was detected in the FL-1 channel using logarithmic amplification. Positive cells were defined by a fixed gate and expressed as a percentage of the total cell population.

Statistical analyses

Results are presented as mean ± SEM. The significance of the differences between groups was analysed with either a one-way or two-way ANOVA followed by a Bonferroni correction or with the Students t-test where applicable, using GraphPad Prism 5; p < 0.05 was considered as significant.

Results

AMPK activation

To determine whether the two pharmacological substances AICAR and ZMP could exert physiological effects via activation...
Javaux et al. reported in 1995 that rabbit cardiomyocytes were unable to phosphorylate AICAR to ZMP. Because of the observed inability of AICAR to elicit glucose uptake in rat cardiomyocytes, we tested the ability of ZMP to affect glucose uptake. As seen in Fig. 2A, ZMP also significantly lowered basal glucose uptake levels (0.6 ± 0.22-fold, \( p < 0.05 \)). To ascertain that this was not because AICAR or ZMP influenced cell viability, PI staining was performed at the end of the experimental protocol. Fig. 2B shows that cell viability was not affected by either substance.

**GLUT4 translocation in cardiomyocytes**

Because of the observed activation of AMPK by AICAR and ZMP, but not of glucose uptake, we assessed GLUT4 translocation under these conditions, fractionating the cells into cytosolic and membrane compartments, and then probing Western blots of the separated proteins with a specific GLUT4 antibody. As shown in Fig. 3A and B, a significant increase in GLUT4 could be seen between basal and stimulated cells in the membrane compartment (1.01 ± 0.02 arbitrary densitometry units vs insulin 1.45 ± 0.2, AICAR 1.29 ± 0.10 and ZMP 1.56 ± 0.1).

**Glucose uptake**

Insulin (100 nM) increased glucose uptake significantly [7.0 ± 0.71-fold, \( p < 0.05 \)] from basal levels in the cardiomyocytes, whereas AICAR (1 mM) diminished glucose uptake 0.6 ± 0.1-fold (\( p < 0.05 \)) from basal levels (Fig. 2A).
results clearly demonstrate that AICAR stimulation of GLUT4 translocation resulted in a protein not exposed on the outside of the cell. In addition, it was demonstrated that insulin treatment led to exposure of GLUT4 on the outside of the cardiomyocyte while AICAR treatment, in accordance with the diminished glucose uptake seen in Fig. 2A, attenuated the amount of protein that could be recognised by the antibody on the outer surface of the cell.

It is postulated that activation of PI-3-kinase or PKB/Akt plays an important role in the docking and fusion of GLUT4 vesicles in insulin-stimulated glucose uptake.31,42 However, neither AICAR nor ZMP resulted in phosphorylation of PKB/Akt (results not shown).

It has also been described that nitric oxide (NO) is important in AMPK-mediated glucose uptake and GLUT4 translocation.43 Because myocytes produce much less NO than endothelial cells,44 we tested the effects of an NO donor, sodium nitroprusside (SNP) in combination with AICAR on the exposure of GLUT4 on the outside of cardiomyocytes, using the flow cytometric method. As shown in Fig. 4, SNP (100 µM) had no effect on the number of myocytes with the exofacial loop of GLUT4 exposed on the outside under control conditions. However, giving SNP together with AICAR led to enhanced exposure of GLUT4 on the outer surface of the cell. Contrary to expectation, this was not accompanied by enhanced glucose uptake (Fig. 5).

Discussion

In this study we aimed to determine whether the pharmacological substance, AICAR, known to activate AMPK in skeletal muscle, also exerted similar effects on AMPK activation, glucose uptake and GLUT4 translocation in isolated, adult ventricular cardiac myocytes. Our results showed significantly increased AMPK phosphorylation of Thr172 in these cells after stimulation with AICAR (Fig. 1), corroborating findings in EDL skeletal muscle45 and hypothalamic cells.46 However, Longnus et al.47 were unable to detect AMPK activation with AICAR in ventricular tissue. In view of the conclusion of Javaux et al.48 that in cardiomyocytes, AICAR is probably not phosphorylated to ZMP, we similarly tested the effect of ZMP and found increased phosphorylation of Thr172 also by this substance. Therefore, both AICAR and ZMP can increase phosphorylation of AMPK in isolated cardiomyocytes.

An increase in AMPK activity leads to stimulation of glucose uptake in skeletal muscle.49,50 However, the significant AMPK phosphorylation noted in our study was not accompanied by a concomitant increase in cardiomyocyte glucose uptake (Fig. 2A). On the contrary, there was a significant decrease in glucose uptake seen in both the AICAR- and ZMP-treated cells. This finding underscores the work by Jessen et al.51 which showed that basal glucose transport in AICAR-exposed animals was significantly lower in all muscles when compared to controls or exercised animals. Additionally, Al-Khalili et al.52 found that both chronic and short-term exposure to AICAR induced AMPK activation in primary human skeletal myocytes but no subsequent increase in glucose uptake.

In contrast to the above, Russell et al.,53 using a slightly longer incubation time, showed that in heart papillary muscle, incubations with AICAR increased glucose uptake almost twofold and led to AMPK phosphorylation and GLUT4 translocation. Papillary muscle, of course, also contains endothelial and endocardial cells.

Both insulin25,34 and AMPK52,54 stimulate glucose uptake by translocation of GLUT4 to the cell membrane. In view of the findings of Russell and co-workers,14 we quantified GLUT4 movement to the cell membrane after various stimuli. Fractionating cells into cytosol and sarcosomal membranes, insulin, AICAR and ZMP treatment resulted in significantly more GLUT4 associated with the cell membrane (Fig. 3). Therefore neither AICAR nor ZMP could stimulate glucose uptake in isolated cardiomyocytes, whereas both substances were able to phosphorylate AMPK and elicit translocation of the GLUT4 transporter from the cytosol to the cell membrane.

The concept that GLUT4 translocation and activation to transport glucose are two independent although interrelated occurrences that can be separated from one another has been put forward by Furtado et al.55 To substantiate this statement, it was demonstrated that intracellular delivery of PIP, results in GLUT4...
translocation and incorporation into the membrane, while no associated glucose uptake was detected. In addition, Funaki and co-workers created a cell-permeable phosphonosidite-binding peptide that could induce GLUT4 translocation to the plasma membrane in adipocytes without increasing glucose uptake.

We used an antibody directed against the exofacial loop of the GLUT4 protein, coupled to a flow cytometric method to determine the amount of GLUT4 exposed on the outer surface of cardiocytes after stimulation with either insulin or AICAR. This method clearly demonstrated enhanced exposure with insulin but attenuated exposure with AICAR, similar to the profile of glucose uptake (Fig. 4). As this was in contrast to the results obtained by Davey et al. showing that ischaemia causes a marked translocation of GLUT4 to the sarcolemmal membrane in whole beating hearts, we speculated on the possible role of NO in this process.

AMPK phosphorylates endothelial nitric oxide synthase (eNOS) on Ser1177. According to Li et al., this activation modulates glucose uptake and GLUT4 translocation in heart muscle. Although cardiomyocytes also contain NOS, isolated cardiocytes produce very little NO in comparison to cardiac endothelial cells. We therefore argued that the lack of NO formation and subsequent activation of the cGMP pathway may be responsible for the lack of glucose uptake in our cells. To test this, we supplemented the cells with NO with the addition of SNP. This demonstrated that indeed, simultaneous stimulation with SNP and AICAR resulted in significantly more GLUT4 protein now detectable with the antibody against the exofacial loop of the protein. However, contrary to our expectations, glucose uptake was not affected, underscoring the concept that there still has to be activation of the GLUT4 transporter after insertion into the membrane. These results then reinforce the finding of Li et al. that more signals than NO in addition to AMPK are necessary to induce glucose uptake.

The results obtained in this study therefore argue that other, hitherto unidentified factors besides AMPK activation and GLUT4 translocation are necessary to induce glucose transport via this pathway. We furthermore concluded that these signals were not activated in the isolated cardiomyocytes via activation of AMPK, although they were active in beating cardiac muscle. Both AICAR and ZMP have proved valuable tools in this study.

References


