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Upregulation of Na^+/H^+ exchanger by the AMP-activated protein kinase

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ABSTRACT

AMP-activated protein kinase (AMPK) is activated upon energy depletion and serves to restore energy balance by stimulating energy production and limiting energy utilization. Specifically, it enhances cellular glucose uptake by stimulating GLUT and SGLT1 and glucose utilization by stimulating glycolysis. During O_2 deficiency glycolytic degradation of glucose leads to formation of lactate and H^+, thus imposing an acid load to the energy-deficient cell. Cellular acidification inhibits glycolysis and thus impedes glucose utilization. Maintenance of glycolysis thus requires cellular H^+ export. The present study explored whether AMPK influences Na^+/H^+ exchanger (NHE) activity and/or Na^+-independent acid extrusion. NHE1 expression was determined by RT-PCR and Western blotting. Cytosolic pH (pH_i) was estimated utilizing BCECF fluorescence and Na^+/H^+ exchanger activity from the Na^+-dependent re-alkalinization (ΔpH_i) after an ammonium pulse. As a result, human embryonic kidney (HEK) cells express NHE1. The pH_i and ΔpH_i in those cells were significantly increased by treatment with AMPK stimulator AICAR (1 mM) and significantly decreased by AMPK inhibitor compound C (10 μM). The effect of AICAR on pH_i and ΔpH_i was blunted in the presence of the Na^+/H^+ exchanger inhibitor cariporide (10 μM), but not by the H^+ ATPase inhibitor bafilomycin (10 nM). AICAR significantly enhanced lactate formation, an effect significantly blunted in the presence of cariporide. These observations disclose a novel function of AMPK, i.e. regulation of cytosolic pH.

1. Introduction

The AMP-activated protein kinase (AMPK), a serine/threonine kinase activated upon increase of the cytosolic AMP/ATP concentration ratio, senses the energy status of the cell [1–3]. AMPK influences cellular glucose uptake, glycolysis, fatty acid oxidation and enzymes required for ATP production [3–6] in an attempt to enhance cellular ATP generation [7]. In addition, AMPK inhibits energy-utilizing mechanisms including protein synthesis, gluconeogenesis and lipogenesis [3,4,7]. Owing to those effects AMPK supports cell survival during energy deprivation [7–9].

AMPK enhances cellular glucose uptake by stimulation of passive, GLUT-mediated transport [10–21] and SGLT1-mediated Na^+-coupled transport [22]. During ischemia glucose is mainly utilized by anaerobic glycolysis, which is similarly activated by AMPK [23–25]. Energy generation by glycolysis is expected to interact with H^+ homeostasis, as anaerobic glycolysis imposes an H^+ load on the cell by generating lactic acid and, conversely, since rate-limiting enzymes of glycolysis are highly pH-sensitive and inhibited by cytosolic acidification [26]. Accordingly, glycolysis can only be maintained, as long as the generated H^+ ions are extruded.

The present study thus explored whether the metabolic-sensing kinase AMPK influences H^+-extruding mechanisms.

2. Materials and methods

2.1. Cell culture and treatments

Experiments were performed in human embryonic kidney (HEK) cells cultured in Dulbecco's MEM, maintained in the presence of 10% FCS and 1% Pen/Strep. AICAR was dissolved in culture medium, cariporide in distilled water, bafilomycin in ethanol and compound C in dimethylsulfoxide (DMSO).

2.2. Measurement of intracellular pH

For digital imaging of cytosolic pH_i the cells were incubated in an HEPES-buffered Ringer solution containing 10 μM BCECF-AM (Molecular Probes, Leiden, The Netherlands) for 15 min at 37 °C. After loading, the chamber was flushed for 5 min with Ringer solution to remove any deesterified dye sticking to the outside of the glands. The perfusion chamber was mounted on the stage of an inverted microscope (Zeiss Axiovert 135), which was used in the epifluorescence mode with a 40× oil immersion objective (Zeiss

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Neoplan, Germany). BCECF was successively excited at 490/10 and 440/10 nm, and the resultant fluorescent signal was monitored at 535/10 nm using an intensified charge-coupled device camera (Proxitronic, Germany) and specialized computer software (Metafluor, USA). The results from each cell were averaged and taken for final analysis. Intensity ratio (490/440) data were converted into pH values using the high-K^+/nigericin calibration technique [27]. Cells were perfused at the end of each experiment for 5 min with standard high-K^+/nigericin (10 μg/ml) solution (pH 7.0). The intensity ratio data thus obtained were converted into pH values using the r_{max}, r_{min}, pK_a values previously generated from calibration experiments (pH range 5–8.5).

For acid loading, cells were transiently exposed to a solution containing 20 mM NH_4Cl leading to initial alkalinization of cytosolic pH (pH_i) due to entry of NH_3 and binding of H^+ to form NH_4^+ [28]. The acidification of cytosolic pH upon removal of ammonia allowed calculating the mean intrinsic buffering power (β) of the cells [28]. Assuming that NH_4^+ and NH_3 are in equilibrium in cytosolic and extracellular fluid and that ammonia leaves the cells as NH_3:

$$\beta = \Delta[NH_4^+]_i/\Delta pH_i$$

where ΔpH_i is the decrease of cytosolic pH (pH_i) following ammonia removal and $\Delta[NH_4^+]_i$ is the decrease of cytosolic NH_4^+ concentration, which is identical to the concentration of $[NH_4^+]_i$ immediately before the removal of ammonia. The pK for NH_4^+/NH_3 is 8.9 [29] and at an extracellular pH (pH_0) of 7.4 the NH_4^+ concentration in extracellular fluid ($[NH_4^+]_0$) is 19.37 mM $[20/(1+10^{pH_0-pK})]$. Intracellular NH_4^+ concentration ($[NH_4]_i$) was calculated from $[NH_4]_i = 19.37\times 10^{pH_0-pH_i}$. The calculation of the buffer capacity required that NH_4^+ exits completely. After the initial decline, pH_i indeed showed little further change in the absence of Na^+, indicating that there was no relevant further exit of NH_4^+. To calculate the ΔpH/min during realkalinization, a manual linear fit was placed over a narrow pH range (pH 6.7–6.9) which could be applied to all measured cells.

The solutions were composed of (in mM): standard Hepes: 115 NaCl, 5 KCl, 1 $CaCl_2$, 1.2 $MgSO_4$, 2 NaH_2PO_4, 10 glucose, 32.2 Hepes; sodium free Hepes: 132.8 NMDG, 3 KCl, 1 $CaCl_2$, 1.2 $MgSO_4$, 2 KH_2PO_4, 32.2 Hepes, 10 mannitol, 10 glucose (for sodium free ammonium chloride 10 mM NMDG and mannitol were replaced with 20 mM NH_4Cl); high K^+ for calibration 105 KCl, 1 $CaCl_2$, 1.2 $MgSO_4$, 32.2 Hepes, 10 mannitol, 10 μg/ml nigericin. The pH of the solutions was titrated to 7.4 or 7.0 with HCl/NaOH, HCl/NMDG and HCl/KOH, respectively, at 37 °C. Where indicated, the K^+ concentration was increased to 35 mM at the expense of Na^+/NMDG.

2.3. Real-time reverse transcription-PCR (RT-PCR)

Total RNA was extracted from HEK cells in Trizol (Peqlab, Erlangen, Germany) according to the manufacturer's instructions. After DNAse digestion reverse transcription of total RNA was accomplished using random hexamers (Roche Diagnostics, Penzberg, Germany) and SuperScriptII reverse transcriptase (Invitrogen, Carlsbad, CA, USA). Polymerase chain reaction (PCR) amplification of the respective genes was set up in a total volume of 20 μl using 40 ng of cDNA, 500 nM forward and reverse primer and 2X iTaq SYBR Green (Bio-Rad, Hercules, CA, USA) according to the manufacturer's protocol. Cycling conditions were performed as follows: initial denaturation at 95 °C for 2 min, followed by 40 cycles of 95 °C for 15 s, 55 °C for 15 s and 68 °C for 20 s. For the amplification the following primers were used (5′→3′ orientation): NHE1 fw AGAGCCACCACGAGAACGCT, rev TGCCATGATCAGTGACGGAAT; TBP fw GCCCGAAACGCCGAATAT, rev CCGTGGTTCGTGGCTCTCT.

The specificity of PCR products was confirmed by analysis of a melting curve. Real-time PCR amplifications were performed on a CFX96 Real-time System (Bio-Rad) and all experiments were performed in duplicate. The house-keeping gene TBP was amplified to standardize the amount of sample RNA. Relative quantification of gene expression was achieved using the Δc_t method as described earlier [30].

2.4. Biotinylation and Western blot analysis

A total of 10^9 HEK cells were biotinylated with EZ-link Sulfo-NHS-Biotin (Pierce protein research products, Thermo Scientific) for 30 min at 4 °C. Then, the cells were washed three times. The cells were lysed with cell lysis buffer followed by separation of the biotinylated proteins with NeutrAvidin Agarose (Thermo Scientific) for 1 h at room temperature. The NeutrAvidin Agarose was washed three times and the NeutrAvidin agarose-bound protein was separated by addition of SDS–PAGE sample buffer. The samples were boiled for 10 min. The biotinylated protein was subjected to 10% SDS–PAGE gel electrophoresis. Proteins were transferred to a PVDF membrane (Millipore Corp.), and the membranes were then blocked for 1 h at room temperature with 5% non-fat dried milk in phosphate-buffered saline containing 0.1% Tween 20. Incubation with the primary anti-NHE1 antibody (1:500, rabbit polyclonal antibody, MBL, Naka-ku Nagoya, Japan) and anti-β actin antibody (1:1000, Cell Signalling Technology, Danvers, MA, USA) was carried out at 4 °C overnight. Specific protein bands were visualized after subsequent incubation with a 1:2000 dilution of anti-rabbit IgG antibody (Dako, Hamburg, Germany) conjugated to horseradish peroxidase and a Super Signal Chemiluminescence detection procedure (Amersham Biosciences). The band intensity was determined by Quantity one software (Biorad gel doc system, Chemidoc XRS).

2.5. Determination of lactate production

Lactate production was measured by slight modification of a previously described method to suit the HEK cells [31]. Briefly, 1×10^6 cells per well were taken in a six-well plate and incubated with AICAR (1 mM) in the presence and absence of cariporide (10 μM) for 24 h at 37 °C in a CO_2 incubator. After 24 h, the cells were scrapped and disrupted by sonication for 5 min and then centrifuged at 15,800 *g* for 10 min. The supernatant was collected and the total lactic acid content in the supernatant was measured by a commercial lactate assay kit (Bioassay systems) according to the manufacturer's instructions.

2.6. Statistical analysis

Data are provided as arithmetic means ± SEM, *n* represents the number of independent experiments. Data were tested for significance using ANOVA or *t*-test, as appropriate. Results with $p < 0.05$ were considered statistically significant.

3. Results

In a first series of experiments the expression of the Na^+/H^+ exchanger (NHE1) was determined in human embryonic kidney (HEK) cells. Real-time PCR was utilized to determine the NHE1 transcript levels. HEK cells were either left untreated or treated with AICAR (1 mM) to activate the AMPK. As shown in Fig. 1a, the expression of NHE1 was significantly enhanced following AICAR treatment. As illustrated in Fig. 1b, Western blotting of biotinylated membrane proteins disclosed that AICAR similarly enhanced the NHE1 protein abundance in the cell membrane.

Increased NHE1 membrane abundance leads to increased Na^+/H^+ exchange in the membrane. To illustrate the increased NHE activity in AICAR-treated cells, the cells were acidified by an