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5-Amino-4-imidazolecarboxamide Riboside Confers Strong Tolerance to Glucose Starvation in a 5′-AMP-Activated Protein Kinase-Dependent Fashion

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Acadesine, 5-amino-4-imidazolecarboxamide riboside (AICAR), has been claimed to protect the heart, lung, and small intestine against ischemic damage. The biochemical mechanisms of this effect of AICAR are not yet fully understood. To understand the mechanism, we examined the effect of AICAR on glucose starvation, since cellular responses to ischemia could be regarded as a protective response to an insufficient blood supply, cells might display adaptive reactions not only to oxygen deficiency but to nutrient deficiency. AICAR was found to confer strong tolerance to glucose starvation. By using antisense RNA expression vector for α subunit of 5′-AMP-activated protein kinase, the effect of AICAR was found to be dependent on 5′-AMP-activated protein kinase containing the α2 subunit. The AICAR effect was also dependent on the presence of amino acids, indicating an energy source switch from glucose to amino acids.

Acadesine, 5-amino-4-imidazolecarboxamide riboside (AICAR), has been claimed to protect the heart, lung and small intestine against ischemic damage (1–3). Several clinical trials have been conducted to evaluate its beneficial effect in preventing adverse effects of coronary artery bypass grafting surgery, and the results, while not dramatic, have been significant (4). The biochemical mechanisms of this effect of AICAR are not yet fully understood. AICAR was first claimed to increase adenosine availability in ischemic myocardium, possibly due to its inhibitory effect on adenosine deaminase, 5′-nucleotidase, and adenosine kinase (5). However, the exact mechanism of the increase in adenosine availability remains to be elucidated.

AICAR was recently found to be a strong activator of 5′-AMP-activated protein kinase (AMPK) after being phosphorylated to form ZMP (6–9). 5′-AMP-activated protein kinase (AMPK) is a heterotrimeric serine threonine protein kinase that is phylogenically conserved from yeast to mammals (10). It is postulated to be a fuel gauge because it is activated by excess concentration of 5′-AMP which is a sensitive marker of decreases in ATP concentration (11), and thus it is activated when cells are exposed to various stressful conditions in terms of their energy status (12). A number of targets of AMPK are known, including glucose transporter, glucose synthetase kinase 3, acyl-CoA carboxylase, and HMG-CoA reductase. The β-subunit of AMPK exhibits a strong similarity to yeast Gal83p, Sip1p and Sip2p, transcription factors, and AMPK is therefore assumed to play a critical role not only in directly maintaining energy production but in regulating gene expression under various stress conditions related to energy supply (13).

We recently found that hypoxia and/or nitric oxide treatment confers strong tolerance against cell death induced by glucose starvation (Esumi *et al.*, to be published elsewhere). Since cellular responses to hypoxia could be regarded as a protective response to an insufficient blood supply in higher organisms, cells might display adaptive reactions not only to oxygen deficiency but to nutrient deficiency. Because hypoxia and NO decrease intracellular ATP levels, we suspected that increases in AMP levels resulting from the above treatment might trigger cellular responses to nutrient deficiency by activating AMPK. We used an activator of AMPK, AICAR, to address this question and found that it conferred strong tolerance to glucose starvation even under normoxic conditions. This finding may pro-

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vide new insights not only into the mechanism of the tissue preservative effect of acadesine but the mechanism of the cellular response to ischemia, and open up a new area of research in the treatment of vascular disorders, cancer, and organ and tissue transplantation.

MATERIALS AND METHODS

Cell culture and treatment. Human hepatoma cell lines HepG2 and HLE and normal human colonic fibroblasts, HF, established in our laboratory by Kenji Sugiyama were maintained in Dulbecco's modified minimum essential medium (DMEM, Gibco) supplemented with 10% dialyzed fetal calf serum (Sigma). DMEM base (Gibco) supplemented with 10% fetal calf serum was used when glucose was withdrawn. When investigating the effect of amino acids, medium containing only electrolytes and buffer according to the composition of DMEM was prepared, and amino acids were supplemented as reported previously (14). Cell survival was assessed by counting the number of viable cells by the trypan blue dye exclusion method.

ATP concentration. Intracellular ATP concentration was assayed with a kit (Sigma) by the method recommended by the supplier. Briefly, after trypsinization, cells were collected by centrifugation at 4°C for 5 min and lysed with extraction buffer. After sonication, the extract was collected by centrifugation at 10,000*g* for 5 min at 4°C. An aliquot was used for determination of ATP by measuring luciferase activity with a Luminescencer Model AB-2100 (Atto, Tokyo, Japan) using ATP as the standard, and another aliquot was used for the determination of protein concentration.

Construction of antisense RNA expression vectors for 5′-AMP-activated protein kinase α1 and α2. A cDNA coding for a constitutive active fragment of AMPK α1 was obtained by the reverse transcription-coupled polymerase chain reaction using primers having the following sequence: 5′-cctggagaaagatggcgacag-3′ and 5′-gtaaagacagctgagaacttcc-3′, covering the −11th nucleotide to the 965th nucleotide of the α1 subunit of AMPK. The PCR product was cloned into the *Eco*RI site of pcDNA3.1 vector (Stratagene), and antisense RNA expression vector was constructed by inserting the above cDNA into pCR3 vector in an antisense orientation. Full-length cDNA coding the α2 subunit of AMPK was obtained by the reverse transcription-coupled polymerase chain reaction using following primers: sense primer 5′-gaagatggctgagaagcaga-3′, covering from the −4th nucleotide, and antisense primer 5′-aactag-agacagatcaacgg-3′, covering to the 1672nd nucleotide of human α2 subunit. The resulting cDNA for α2 subunit was inserted into the pCR3 vector in an antisense orientation to provide an antisense RNA expression vector. All cDNAs were verified by total sequencing. Transfection of the plasmid into HepG2 cells was achieved by a calcium phosphate coprecipitation method, and selection was achieved with 670 μg/ml G418 for at least 3 weeks.

AMPK activity determination. AMPK activity was determined by an *in vitro* phosphorylation assay using SAMS-GST fusion peptide as a substrate, essentially based on the method of Davies *et al.* (15), with modifications (10, 16) (Kishimoto *et al.*, to be published elsewhere). The details of the method will be published elsewhere, but briefly be described as follows. SAMS-GST fusion peptide was expressed in *E. coli* by transfection with an expression vector. The expression vector was constructed by ligating synthetic nucleotide coding for SAMS peptide (16) into pGEX-4T-1 vector (Amersham-Pharmacia). The fusion peptide was purified by glutathione-Sepharose column chromatography. Cell extracts were obtained by lysing cells with buffer A (10) containing 1% Triton X-100 and centrifugation at 8100*g* for 15 min followed by polyethylene glycol precipitation. Protein was determined by the Bradford method as recommended by the supplier (Bio-Rad).

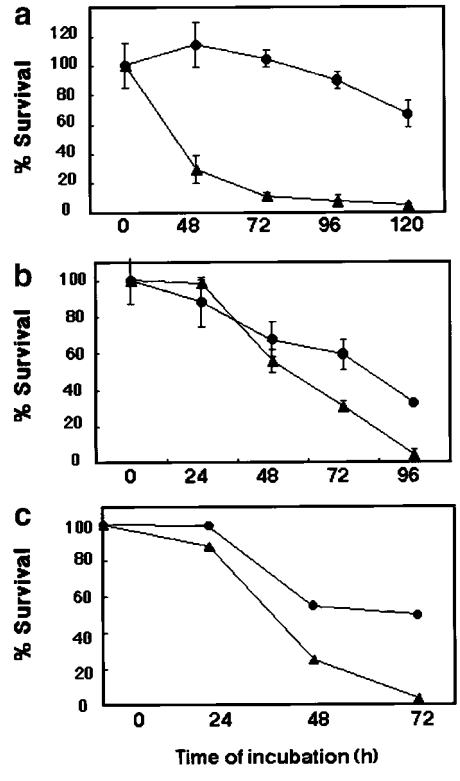

FIG. 1. The effect of AICAR on cell survival during glucose starvation. (a) HepG2 cells were cultured with (●) and without (▲) 0.5 mM AICAR in the absence of glucose. (b) HLE cells were cultured with (●) and without (▲) 1 mM AICAR in the absence of glucose. (c) HF cells were cultured with (●) and without (▲) 0.25 mM AICAR in the absence of glucose.

Northern blot analysis. Total RNA was extracted from cultured cells using acid guanidinium thiocyanate–phenol–chloroform extraction. Twenty micrograms of total RNA per lane was electrophoresed on a 1% agarose, 6.6% formaldehyde denaturing gel and blotted onto nylon membranes. The blots were hybridized with ^{32}P-labeled human AMPK α1 or α2 cDNA, and visualized by exposure to an imaging plate (BAS-2000 system, Fuji Film, Tokyo, Japan). A β-actin probe was used as a control for the amount of RNA in each lane

RESULTS AND DISCUSSIONS

When human hepatoma HepG2 cells were cultured in Dulbecco's modified Eagle minimal essential medium without glucose (DMEM-base, Gibco) supplemented with 10% dialyzed fetal calf serum at atmospheric oxygen concentration under 5% CO_2, most of the cells underwent necrotic cell death within 24 h. However, when 0.5 mM AICAR was included in the culture medium, there was a significant increase in cell survival (Fig. 1). Although on a small scale, a similar increase in cell survival was observed when another human hepatoma cell line, HLE, and human normal colonic fibroblasts, HF, were used (Figs. 1b and 1c).

AICAR is known to undergo phosphorylation to yield ZMP, which is an analogue of AMP, and to activate

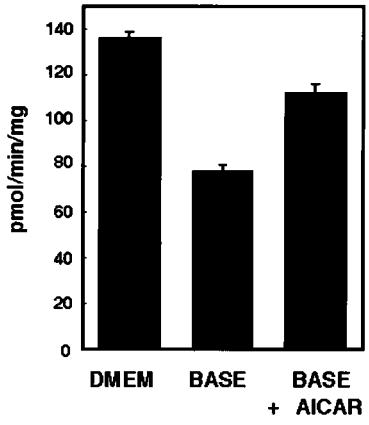

FIG. 2. The effect of AICAR on AMPK activity. HepG2 cells were cultured in either DMEM or DMEM-base with and without 0.5 mM AICAR for 3 h, and AMPK activity was extracted and assayed using SAMS-GST fusion peptide as the substrate. The results are the means of data from 4 samples each. Bars indicate standard errors.

AMP-activated protein kinase (9). The AMPK activity of HepG2 cells after glucose starvation and AICAR treatment was assessed by their ability to phosphorylate SAMS peptide. As shown in Fig. 2, AMPK activity decreased slightly after 5 h glucose starvation, but was maintained by addition of AICAR during glucose starvation. These findings indicated that AICAR actually increased the active form of the AMPK during glucose starvation. Although previous papers have reported that AMPK is activated during glucose starvation, AMPK activity decreased slightly under the present conditions. The reason for this discrepancy is not yet clear but it may be attributable to differences in cell type because different amounts of AMPK isotypes are observed in different types of cells.

Cell survival during tolerance to glucose starvation induced by hypoxia is dependent on certain amino acids (Esumi *et al.*, to be published elsewhere). The conditions for tolerance induced by AICAR were investigated by withdrawing amino acids. Increased cell survival was observed only when amino acids were present, indicating that amino acids are essential to the induction of tolerance by AICAR (Fig. 3a). The number of surviving cells seems to decrease by addition of AICAR when glucose was present. But this is caused not by cell death but by cell cycle delay due to AICAR addition through phosphorylation of p53 (17). Cellular ATP levels were examined under the same conditions, but after 12 h of incubation. The amounts of ATP at 12 h correlated well with cell survival at 48 h of starvation, except for survival during both amino acid and glucose starvation. We do not yet know the reason for this slight discrepancy yet. These findings indicated that maintenance of intracellular ATP levels was an important determinant of cell survival in this experiment.

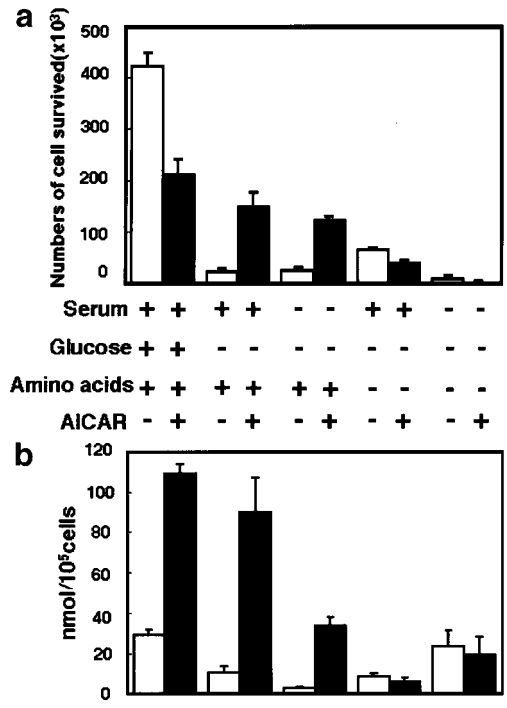

FIG. 3. Cell survival during starvation with respect to various nutrients. (a) HepG2 cells were cultured under various conditions. After 48 h of incubation, the number of surviving cell was counted by the trypan blue dye exclusion method. (b) Intracellular ATP concentration at 12 h of culture. After extraction, HepG2 cells cultured under various conditions were assayed for intracellular ATP concentration.

AMPK is a heterotrimeric enzyme composed of a catalytic α subunit and β and γ subunits, and two isoforms of the α subunit are known in human cells (12). To confirm the involvement of AMPK in AICAR-induced tolerance to glucose starvation, antisense RNA expression vectors for the α1 and α2 subunits were transfected into HepG2 cells. After selection by G418

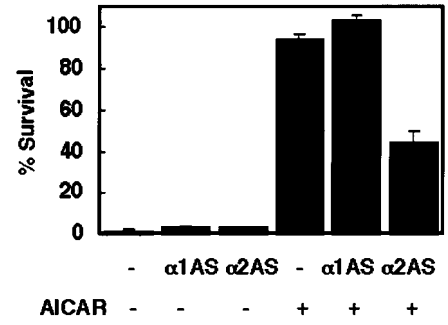

FIG. 4. The effect of antisense RNA expression vectors for AMPK α subunits on HepG2 cell survival in the presence of AICAR without glucose. Cell survival was assessed after 48 h culture with and without 0.5 mM AICAR without glucose. α1AS, HepG2 cells stably transfected with antisense expression vector for AMPK α1. α2AS, HepG2 cells stably transfected with antisense expression vector for AMPK α2.

at 640 μg/ml for more than 3 weeks, respective mixed cultures of stable resistant cells were obtained, and these cells were subjected to glucose starvation in the absence and presence of AICAR for 48 h. As shown in Fig. 4, AICAR clearly induced tolerance to glucose starvation in parental HepG2 cell and α1-antisense RNA expression vector transfected HepG2 cells but significantly less tolerance was observed in the α2-antisense RNA expression vector transfected HepG2 cells. These findings clearly indicate that the induction of tolerance by AICAR is dependent on activation of AMPK consisting of α2 subunit, and this observation is highly consistent with the previous notion that the α2 subunit but not the α1 subunit, is sensitive to AMP (18). Expression of α1 and α2 isoforms in a different cell line was examined to further confirm this. As expected, expression of the α2 isoform was higher in the HepG2 cells than in the HLE and HF, in which the protective effects of AICAR were less than in HepG2 (Fig. 5).

The hypoxia response plays an important role in both circulatory and pulmonary diseases and cancer. The HIF-1 transcription factor is known to be a key mediator of these reactions, including the switch to anaerobic metabolism and improvement of oxygen supply, angiogenesis, and erythropoiesis. We therefore suspected that HIF-1 might be the target of AMPK and investigated this possibility by EMSA. As shown in Fig. 6, however, no effect of AICAR was observed either under normoxic or hypoxic conditions, or with or without glucose.

The results of the present study clearly show that AICAR confers strong tolerance to glucose starvation in various cells. This induction of tolerance is dependent on the AMPK a2 subunit. Although differential expression of AMPK isoforms has been reported, it has not been studied extensively (11, 13, 19–22). The protective effect of AICAR is somewhat controversial depending on the experimental system. The difference in isoform expression of AMPK might be attributable to the difference in the responses of tissues and cells to AICAR (23). The physiological relevance of the present finding is still hypothetical, but it seems to be related to the response to ischemia. If so, the present findings provide new insight not only into the molecular mechanisms of the effect of AICAR but into the ischemic response directly related to the pathophysiology of vascular disorders and cancer and tissue and organ transplantation.

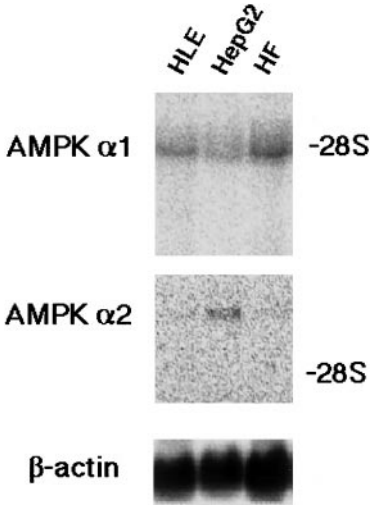

FIG. 5. Northern blot analyses of AMPK α1 and α2 mRNAs. Expression of AMPK α subunit mRNA was examined by Northern blots using the respective specific probe for α1 and α2 in three cell lines tested for their tolerance to glucose starvation induced by AICAR.

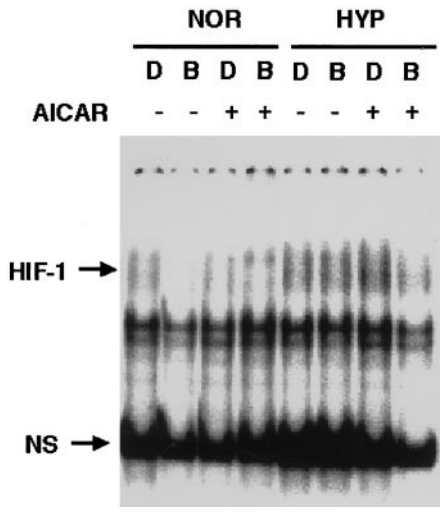

FIG. 6. The effect of AICAR treatment on HIF-1 activation. HepG2 cells were cultured in the presence (D) and absence (B) of glucose with and without 0.5 mM AICAR under normoxic (NOR) and hypoxic (HYP) conditions. D, DMEM; B, DMEM-base. NS, nonspecific binding.

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