AMP-activated Protein Kinase Is Involved in Neural Stem Cell Growth Suppression and Cell Cycle Arrest by 5-Aminoimidazole-4-carboxamide-1-β-D-ribofuranoside and Glucose Deprivation by Down-regulating Phospho-retinoblastoma Protein and Cyclin D*

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The fate of neural stem cells (NSCs), including their proliferation, differentiation, survival, and death, is regulated by multiple intrinsic signals and the extrinsic environment. We had previously reported that 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR) directly induces astroglial differentiation of NSCs by activation of the Janus kinase (JAK)/Signal transducer and activator of transcription 3 (STAT3) pathway independently of AMP-activated protein kinase (AMPK). Here, we reported the observation that AICAR induced G1/G0 cell cycle arrest in NSCs, associated with decreased levels of poly(ADP-ribose) polymerase, phospho-retinoblastoma protein (Rb), and cyclin D but did not cause apoptosis. Iodotubericidin and Compound C, inhibitors of adenosine kinase and AMPK, respectively, or overexpression of a dominant-negative mutant of AMPK, but not JAK inhibitor, were able to reverse the anti-proliferative effect of AICAR. Glucose deprivation also activated the AMPK pathway, induced G0/G1 arrest, and suppressed the proliferation of NSCs, an effect associated with decreased levels of phospho-Rb and cyclin D protein. Furthermore, Compound C and overexpression of dominant-negative AMPK in C17.2 NSCs could block the glucose deprivation-mediated down-regulation of cyclin D and partially reverse the suppression of proliferation. These results suggest that AICAR and glucose deprivation might induce G1/G0 cell cycle arrest and suppress proliferation of NSCs via phospho-Rb and cyclin D down-regulation. AMPK, but not JAK/STAT3, activation is key for this inhibitory effect and may play an important role in the responses of NSCs to metabolic stresses such as glucose deprivation.

Neural stem cells (NSCs), defined by their ability to self-renew and differentiate into the three major cell types, neurons, astrocytes, and oligodendrocytes, play an essential role in the development and maturation of the nervous system. They exist not only in the developing brain but also in the adult brain in several areas with neurogenic potential (1). Self-renewal is regulated by a dynamic interplay between transcription factors, epigenetic control, microRNA regulators, and cell-extrinsic signals from the microenvironment in which NSCs reside. Among cell-intrinsic signals, nuclear receptors such as orphan nuclear receptor TLX (2), estrogen receptors (3–5), thyroid hormone receptors (6), and peroxisome proliferator-activated receptor γ (7) have been shown to be essential transcriptional regulators of NSC maintenance and self-renewal in the adult brain. Polycomb transcriptional repressor Bmi1, high mobility group DNA-binding protein Sox2, and basic helix-loop-helix transcription factors also play central roles in regulation of NSC maintenance throughout development and adulthood (8–10). As for NSC lineage-specific cell-intrinsic signals, Notch (11), Wnt/β-catenin (12), basic fibroblast growth factor (13), epidermal growth factor (14), bone morphogenetic protein (15), transforming growth factor-α (16), and Sonic hedgehog (17), signaling pathways have also been implicated in the control of self-renewal of NSCs and may act as important niche factors to maintain them.

Because of their ability to self-renew, NSCs have promise for treatment of neurological diseases by transplantation therapy or by recruitment of endogenous precursors to repair the adult brain. In contrast, it has been reported that NSCs and brain tumors share many common features (18). They express common sets of markers (e.g. Nestin, CD133, and Sox2) and share similar pathways regulating their proliferation (e.g. Sonic

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§ The abbreviations used are: NSC, neural stem cells; JAK, Janus kinase; STAT3, signal transducer and activator of transcription 3; AMPK, AMP-activated protein kinase; DN-AMPK, dominant-negative AMPK; AICAR, 5-aminimidazole-4-carboxamide-1-β-D-ribofuranoside; ZMP, AICAR monophosphate; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; ITU, iodotubericidin; EGFP, enhanced green fluorescent protein; STS, staurosporine; EGF, epidermal growth factor; CNTP, cilary neutrophic factor; ACC, acetyl CoA carboxylase; Rb, retinoblastoma protein; PBS, phosphate-buffered saline; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid; PARP, poly(ADP-ribose) polymerase; FACS, fluorescence-activated cell sorter.
AMPK Activation Inhibits Proliferation of Neural Stem Cells

hedgehog, phosphatase, and tensin homolog). Study of the self-renewal of adult NSCs will yield new insights into brain cancer (19–21), including further understanding of NSC origins and use in clinical therapy.

Recently, we demonstrated that a cell-permeable small molecule, 5-aminoimidazole-4-carboxamide-1-β-d-ribofuranoside (AICAR) could directly induce astroglial differentiation of NSCs. AICAR is a very commonly used and quite specific activator (22) of AMP-activated protein kinase (AMPK), a metabolic master regulator (23, 24). It has also been reported to have diverse effects on lipid and glucose metabolism, regulation of the pro-inflammatory response, and cytokine production, most of which are associated with AMPK activation. However, our recent study showed that the effect of AICAR on induction of astroglial differentiation of NSCs is independent of its classical target AMPK but dependent on JAK/STAT3.

In conjunction with this effect on astrogliogenesis, we also observed a reduction in the number of NSCs. Although AICAR has been reported to regulate cell proliferation, induce cell cycle arrest, and regulate apoptosis in various cell types, including a protective anti-apoptotic effect in astrocytes (25) and hippocampal neurons (26), the reported details of these effects and their underlying mechanisms are varied and intricate. Thus far, there are few reports of the effects of AICAR or any other metabolic regulator on the self-renewal of NSCs or other stem cells. In the present study we investigated the mechanism by which AICAR exposure reduces NSC number and the role of AMPK and the JAK/STAT3 pathway in the antiproliferative effect of AICAR in NSCs. Finally, we investigated the role of AMPK in NSC survival under conditions of cellular stress induced by depletion of energy such as glucose deprivation.

MATERIALS AND METHODS

Chemicals—AICAR, d-mannose, d-glucose, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and propidium iodide were purchased from Sigma-Aldrich. JAK inhibitor I, Compound C, staurosporine (STS), and iptodutibericidin (ITU) were from Calbiochem.

Neural Stem Cell Culture—Mouse immortalized NSC line C17.2 (C17.2-NSC, a generous gift from Dr. Tie-Qiao Wen) was originally described by Snyder et al. (27). The C17.2-NSC were maintained in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal calf serum (Hyclone), 5% horse serum (Invitrogen), and 2 mM glutamine (Invitrogen) in a humidified atmosphere of 5% CO2 and 95% air at 37 °C and split when the cells reached 90% confluency. Primary NSCs were isolated from the cerebral cortices of embryonic day 14 (E14) SD. rats according to methods described previously (28), and all experiments were performed with cells that had undergone two passages.

The primary NSCs (E14-NSC) were maintained on uncoated 25-ml flasks (Corning) in Dulbecco’s modified Eagle’s medium (Invitrogen) that contained N2 and B27 supplements (Invitrogen) plus penicillin (Sigma, 100 μg/ml), streptomycin (Sigma, 100 μg/ml), basic fibroblast growth factor (Promega, 10 ng/ml), and epidermal growth factor (Promega, 20 ng/ml). For monolayer growth of E14-NSC, the cells were mechanically dissociated from the E14 neurospheres and plated at a density of 20 × 10^4 cells/ml on 100 μg/ml poly-d-lysine (Sigma)-coated glass coverslips or 6-well plates (Costar). The monolayers were maintained in Dulbecco’s modified Eagle’s medium/F-12 medium (Invitrogen) that contained N2 and B27 supplement (Invitrogen) plus penicillin (100 μg/ml, Sigma), streptomycin (100 μg/ml, Sigma), basic fibroblast growth factor (20 ng/ml, Promega), and epidermal growth factor (20 ng/ml, Promega). Medium was supplemented with basic fibroblast growth factor and epidermal growth factor each day.

Plasmid Construction and Transfection—cDNA encoding human AMPK-α1, containing a mutation that alters aspartic acid 159 to an alanine (D159A) (29), was subcloned into the pCAGGS-IRE-EGFP vector (a kind gift from Dr. Ding YQ of Shanghai institute for biological sciences) to generate pCAGGS-DN-AMPK-IRE-EGFP. Lipofectamine™ 2000 (Invitrogen) was used for transfection as directed by the manufacturer.

Western Blot Analysis—20–100 μg of protein per lane was loaded onto a 10% SDS-polyacylamide gel and then transferred to a Hybond-C nitrocellulose membrane (Amersham Biosciences). The membranes were processed for immunoblotting as described previously (28). The following primary antibodies were obtained from Cell Signaling Technology and used at 1:1000 dilutions unless otherwise indicated: AMPK-α, phospho-AMPKα (Thr-172), ACC, phospho-ACC (Ser-79), STAT3, phospho-STAT3 (Tyr-705), phospho-Rb (Ser-807/811), cyclin D, and anti-actin (Sigma, 1:5000). Rb (1:1000) was purchased from ProteinTech Group, Chicago, IL. Horseradish peroxidase-labeled anti-rabbit or anti-mouse secondary antibody (1:5000) was purchased from Jackson ImmunoResearch. Immunoreactive bands were visualized by enhanced chemiluminescence (Amersham Biosciences).

Trypan Blue Exclusion Test—Cells were harvested with trypsin, and a small aliquot of the cell suspension was diluted with an equal volume of 0.4% trypsin blue solution. Cells were counted under a phase-contrast microscope. Cells with a damaged cell membrane (necrotic cells) stained blue, whereas cells with an intact plasma membrane (healthy or apoptotic cells) remained unstained. All experiments were performed in triplicate and were repeated at least three times.

Nuclear Staining with Hoechst 33342—C17.2-NSCs treated with 1 mM AICAR were cultured on poly-d-lysine-coated slides for 48 h. The cells were then fixed with 4% paraformaldehyde in PBS for 10 min and rinsed. Chromatin staining was performed with Hoechst 33342 to detect nuclear condensation (a morphological change associated with apoptosis), and the cells were observed using a fluorescence microscope (IX70, Olympus).

Measurement of Caspase Activity—The caspase activity in the AICAR-treated C17.2-NSCs were detected according to a protocol previously described (30). AICAR-treated cell were washed once using PBS and resuspended in 200 μl of lysis buffer composed of 50 mm Hepes (pH 7.5), 10 mm dithiothreitol, 5 mm EDTA, 10 μg/ml proteinase K, 100 μg/ml phenylmethylsulfonyl fluoride, 10 μg/ml pepstatin, and 10 μg/ml leupeptin. Cells in lysis buffer were cooled to −80 °C and then warmed to 4 °C four times to lyse them completely. The samples were centrifuged at 12,000 × g for 20 min at 4 °C. The protein concentrations of the supernatants were measured using the Bradford method. Caspase-3 activity was measured in a volume of 100 μl...
AMPK Activation Inhibits Proliferation of Neural Stem Cells

RESULTS

AICAR Inhibits Proliferation of Neural Stem Cells—We found a reduction in NSC number accompanying the differentiation induced by AICAR. AICAR exposure can significantly inhibit C17.2 proliferation in a dose-dependent (Fig. 1A) and time-dependent (Fig. 1B) manner compared with the controls. Reductions in C17.2 cell numbers of 5.48, 17.27, 23.79, 35.00, and 76.77% were induced by 48 h of treatment with 0.125, 0.25, 0.5, 1, and 2 mM AICAR, respectively. Treatment with 1 mM AICAR for 24, 48, and 72 h resulted in 6.54, 36.15, and 41.99% reduction of the total cell number, respectively.

AICAR Causes Cell Cycle Arrest in G0/G1 Phase—The reduction in cell number could result from either an increase in cell death or inhibition of proliferation. To distinguish these possibilities, we first investigated the effect of AICAR on cell cycle progression using flow cytometry analysis. Compared with control cells, AICAR treatment for 24 h significantly increased the proportion of C17.2-NSCs in the G0/G1 phase (from 46.58 ± 2.05 to 60.78 ± 0.75%) and decreased that in the S phase (from 36.39 ± 4.33 to 28.98 ± 2.20%) and G2/M phases (from 18.03 ± 3.19 to 10.05 ± 2.17%) (Fig. 2A). This effect of AICAR was highly significant (p < 0.001, n = 3).

As expected, similar results were observed for AICAR-treated primary E14-NSCs (Fig. 2B). The G1 arrest induced by AICAR was obvious in comparison with the untreated controls. The proportion of G0/G1 phase cells increased from 66.96% in controls to 75.28% in AICAR-treated cells, whereas S phase and G2 phase decreased from 15.02 to 10.46% and from 18.03 to 14.26%, respectively.

AICAR Decreases the Phosphorylation of Rb and the Protein Level of Cyclin D in Neural Stem Cells—Phosphorylation of Rb is reported to be a critical and common event during the process of cell proliferation and plays an especially important role in G1 progression and G1-S transition. We next examined the effect of AICAR on Rb. Western blot analysis of the C17.2 cell lysate after 1–2 days of AICAR treatment showed that AICAR dramatically decreased the phosphorylation of Rb in a time-dependent fashion compared with control cells (Fig. 2C). In agreement with this, the protein level of the upstream regulator of Rb, cyclin D, in C17.2-NSCs was also found to decrease in a time-dependent manner. Similar results were observed in the...
AMPK Activation Inhibits Proliferation of Neural Stem Cells

primary E14-NSC, i.e. a decrease of cyclin D protein expression and Rb phosphorylation (Fig. 2D). Our results suggest that the AICAR-induced G<sub>0</sub>/G<sub>1</sub> arrest in NSCs might be associated with decreasing levels of phospho-Rb and D-cyclin protein.

**AICAR Does Not Cause Necrosis or Apoptosis of Neural Stem Cells**—We then investigated whether AICAR could induce cell death (necrosis or apoptosis). In a trypan blue exclusion assay, no difference was observed in the proportion of dead cells between AICAR-treated and vehicle-treated C17.2 cells (data not shown). To determine whether 1 mM AICAR induces apoptosis of NSCs, we stained the nuclei of 1 mM AICAR-treated C17.2 with Hoechst 33342. AICAR-treated C17.2 showed no obvious nuclear condensation compared with negative and positive control cells (Fig. 2E). We then measured the absolute activity of caspase-3 in the cell lysates and observed a slightly increased activity of caspase-3 (about 10 times that in the control cells, Table. 1) in AICAR-treated cells, which is much lower than that induced by 0.25 μM STS, an inducer of apoptosis in NSCs (Fig. 2F). In addition, Western blot analysis showed slightly decreased expression of procaspase-3, which was in accord with its increasing activity, and no obvious accumulation of poly(ADP-ribose) polymerase (PARP) cleavage fragment in AICAR-treated C17.2 cells after 2 days of treatment, whereas STS produced more cleaved PARP fragment (Fig. 2G). Interestingly, we found an evident reduction in full-length PARP after 2 days of treatment with AICAR although no cleavage fragment was produced. Thus, we speculated that AICAR probably down-regulated the expression of PARP, a hypothesis that needs to be confirmed. Our results suggest that AICAR induces a non-apoptotic activation of caspase-3 in NSC. These data indicate that the AICAR-induced reduction in the number of NSC is probably a result of the inhibition of cell proliferation by G<sub>0</sub>/G<sub>1</sub> arrest rather than to cell death.

**Inhibition of AMPK Activity by Inhibitors or Dominant-negative AMPK (DN-AMPK) Blocks the Growth-suppressive Effect of AICAR on NSCs**—AICAR was the first known AMPK activator, and most of its effects have been shown to be the result of AMPK activation. However, in our recent study we found that although AICAR could activate the AMPK pathway in NSC as it does in other cell types, several lines of evidence suggest that this enzyme is not essential in the astrogligenic effect of AICAR. To exclude the possibility that the inhibitory effect of AICAR on NSC proliferation is also caused by mechanisms other than AMPK activation, we investigated the effects of two different inhibitors of AICAR function, ITU and compound C. ITU blocks the conversion of AICAR to its activated phosphorylated form, ZMP, inside the cell by inhibiting an adenosine kinase, and Compound C is a well known AMPK inhibitor. Pretreatment with ITU (0.2 μM)
AMPK Activation Inhibits Proliferation of Neural Stem Cells

FIGURE 3. Effects on the anti-proliferative effect of AICAR of inhibition of AMPK activity by inhibitors or by DN-AMPK. C17.2-NSCs were pretreated with ITU (0.2 μM; A), Compound C (5 μM; B), or vehicle (DMSO) for 1 h. Cells were then incubated for 2 days without or with 1 mM AICAR in the presence of inhibitors. An MTT incorporation assay was performed. The data are the mean ± S.E. (n = 3) from three separate experiments. p < 0.01 (**) and p < 0.05 (*) and are compared with vehicle. C, immunoblot analysis of phospho-Rb and the protein level of cyclin D in the cell lysate of DN-AMPK-transfected C17.2 after 48 h of AICAR treatment. The overexpression of DN-AMPK and the decrease of endogenous AMPK activity by dominant suppression were examined by blotting with anti-pan-AMPK antibody and anti-phospho-ACC antibody in AICAR-treated DN-AMPK/C17.2. Western blot results confirm a dramatic decrease in AMPK activity and also reveal that transfected DN-AMPK abolishes the AICAR-mediated down-regulation of cyclin D and phospho-Rb. The blots are representative of three individual experiments. D, cell cycle analysis of DN-AMPK-transfected C17.2 treated with 1 mM AICAR for 24 h or untreated using pcAGGS-IRES-EGFP vector as a control (mock). After overnight fixation cells were suspended in PBS with RNase A and propidium iodide and then analyzed for DNA content by flow cytometry using ModFit LT software. The FACS analysis indicated that DN-AMPK partially but significantly blocked AICAR-induced cell cycle arrest. The data are representative of three separate experiments performed in triplicate, p < 0.001 (**) and p < 0.05 (*) and are compared with AICAR-treated mock (pcAGGS-IRES-EGFP)-transfected cells.

Completely blocked the inhibitory effect of AICAR on proliferation of NSC (Fig. 3A). Compound C (5 μM) partially but significantly blocked the inhibitory effect of AICAR on NSC proliferation (Fig. 3B). Pretreatment with these inhibitors completely blocked AICAR-induced phosphorylation of AMPK and ACC. These results indicate that ZMP formation through phosphorylation of AMPK and activity of AMPK is required for the suppression of growth by AICAR.

To further confirm the involvement of AMPK in the growth-suppressive effect of AICAR on NSC, we performed experiments using a dicistronic vector simultaneously expressing a dominant-negative form of AMPKα1 (DN-AMPK) and the gene for enhanced green fluorescent protein (EGFP) to mark the transfected cells, as described in our recent paper (28). Overexpression of DN-AMPK, confirmed by Western blotting using anti-pan-α-AMPK antibody, but not of empty vector (mock transfection) suppressed AICAR-induced phosphorylation of ACC (Fig. 3C). In mock-transfected NSC, AICAR decreased cyclin D expression and Rb phosphorylation (Fig. 3C) as shown in Fig. 2. DN-AMPK partially but significantly reversed this down-regulatory effect of AICAR, implying the involvement of AMPK.

Furthermore, FACS analysis showed that in mock-transfected C17.2-NSC AICAR caused obvious G0/G1 arrest, increasing the proportion of cells in G0/G1 phase from 44.43 to 61.21%, as shown in Fig. 3A. DN-AMPK transfection partially but highly significantly (***, p < 0.001, n = 3) blocked cell cycle arrest by AICAR, with the proportion of DN-AMPK-transfected cells in G0/G1 phase being 45.21 and 51.7% after 24 h of DMSO or AICAR treatment, respectively (Fig. 3D). This result is in agreement with those of the Western blot analysis of the growth regulators. Taken together, all of the above evidence clearly demonstrates that AMPK activation is a key process for the inhibitory effect of AICAR on the proliferation of NSC.

The JAK-STAT3 Pathway Is Not Responsible for the Anti-proliferative Effect of AICAR on NSCs—We recently reported that AICAR could activate the JAK/STAT3 pathway in NSC and that its newly reported effect (i.e. inducing astroglial differentiation of NSC) is independent of its classical target AMPK but occurs via activation of the JAK/STAT3 pathway. However, although all of the above evidence shows that AMPK plays a key role in the anti-proliferative effect of AICAR on NSC, we wondered about the role of JAK/STAT3 pathway in this process.

We first investigated the effect of CNTF, a reference activator of the JAK/STAT3 pathway and classical gliogenesis inducer, on the proliferation of NSC. As expected, CNTF induced the phosphorylation of STAT3 (Fig. 4A), whereas it had no obvious effect on NSC proliferation and cyclin D protein level (Fig. 4, B and C). This suggested that there might not be a correlation between the activation of JAK/STAT3 pathway and the growth inhibition of NSC.

We then used a pan-specific JAK inhibitor, JAK inhibitor I, to further determine whether the activation of the JAK/STAT3 pathway contributes to the AICAR-induced growth suppression. C17.2-NSCs were pretreated with various concentrations of JAK inhibitor I (0, 1, 5, 10 μM) 1 h before the addition of 1 mM AICAR, and their proliferation was measured with an MTT assay after 48 h. The results shown in Fig. 4D indicate that JAK inhibitor I treatment had no effect on AICAR-induced NSC inhibition, because the ability of JAK inhibitor I to block...
AMPK Activation Inhibits Proliferation of Neural Stem Cells

AICAR-induced STAT3 phosphorylation had already been confirmed in our recent report (28). These results suggest that the JAK/STAT3 pathway is not essential in the inhibitory effect of AICAR on NSCs.

Low Glucose Activates the AMPK Pathway and Suppresses the Proliferation in NSC—As a metabolite-sensing protein kinase AMPK was reported to play an important role in responses to metabolic stress in muscle cells. Although we recently reported that AMPK is not involved in the AICAR-induced astrogliogenesis of NSCs, all the evidence reported here points strongly toward an important role of AMPK as an effective anti-proliferative system in NSC. Therefore, it was of interest for us to investigate the role of AMPK in NSC survival under conditions of cellular stress induced by depletion of energy. Here, we chose glucose deprivation as the metabolic stimulus.

First, we investigated whether low glucose could stimulate the activation of the AMPK pathway in NSC, as it does in muscle cells. Glucose deprivation was achieved by replacing some or all of the D-glucose in the growth medium with D-mannose, a stereoisomer of glucose that is neither transported into cells nor metabolized by them. The Western blot analysis of NSCs exposed to low glucose (25, 10, 2.5, 0 mM) for 24 h showed that low glucose induced the phosphorylation of AMPK and ACC in a dose-dependent manner (Fig. 5A). We then studied the effect of glucose deprivation on NSC proliferation. As shown in Fig. 5, B and C, low glucose exposure significantly inhibited NSC proliferation in a dose- and time-dependent manner.

We further examined the regulatory effect of low glucose on levels of phospho-Rb and cyclin D, which had been significantly changed by AICAR treatment. Western blot analysis of the cells exposed to medium containing different concentrations of glucose showed that compared with high glucose, glucose deprivation dramatically decreased the phosphorylation of Rb and the protein level of cyclin D in C17.2 in a dose-dependent manner (Fig. 6C). Our results suggest that in NSCs, glucose deprivation causes a G1 arrest associated with down-regulation of Rb phosphorylation and D-cyclin expression in a similar manner to AICAR.

Inhibition of AMPK Activity by Inhibitors or DN-AMPK Partially but Significantly Blocks the Growth-suppressive Effect of Glucose Deprivation on NSCs—To further confirm the role of AMPK in glucose deprivation-induced growth suppression of NSCs, we first used the AMPK inhibitor Compound C as we did in AICAR-treated cells. Pretreatment with Compound C (5 μM) partially but significantly reversed the inhibitory effect of glucose deprivation on NSC proliferation (Fig. 7A). C17.2-NSCs were then transiently transfected with DN-AMPK, as described earlier. As expected, the effectiveness of DN-AMPK overexpression was confirmed by detecting increased AMPK expression and decreased endogenous AMPK activity.

As shown in Fig. 7B, glucose deprivation clearly inhibited the proliferation of mock-transfected C17.2-NSCs, and DN-AMPK partially but significantly (*, p < 0.05, n = 3) blocked glucose deprivation-induced proliferation suppression com-
pared with no obvious difference between the growth rate of mock-transfected and DN-AMPK-transfected C17.2-NSCs.

In addition, glucose deprivation decreased cyclin D expression and Rb phosphorylation in mock-transfected NSCs as observed in Fig. 6C. DN-AMPK partially but significantly reversed this down-regulation, implying the involvement of AMPK (Fig. 7C). All these data demonstrate that AMPK may play an important role in the response of NSCs to glucose deprivation.

**DISCUSSION**

We recently reported that a cell-permeable small molecule, AICAR, was able to alter the fate of NSCs by directly inducing astroglial differentiation. Accompanying the astroglial differentiation, we also found an obvious reduction in cell number. In the present study, we investigated the underlying mechanism of this effect.

We first demonstrated that AICAR induces NSC G0/G1 arrest but not apoptosis or necrosis, as judged by several different experimental approaches involving assays of cell proliferation (Fig. 1) including trypan blue exclusion assay, nuclear morphology (Fig. 2E), caspase-3 activity detection (Fig. 2, F and G, Table 1), and DNA content estimation through FACS analysis (Fig. 2, A and B). In agreement with these findings, the observed down-regulation of cyclin D protein and dephosphorylation of phospho-Rb (Fig. 2, C and D) caused by AICAR strongly indicated that this molecule induces a G1/G0 arrest associated with the cyclin D/Rb signaling pathway, which is suggested to play an important role in NSC proliferation (31, 32).

Although based on the present FACS results we cannot distinguish the cells in G0 phase from those in G1 phase, we believe that AICAR treatment probably induces more NSCs to exit the cell cycle and enter a stage of mitotic quiescence (G0 phase) because of the observed increase in differentiated cells. This
AMPK Activation Inhibits Proliferation of Neural Stem Cells

![Graph](Image)

**FIGURE 7. AMPK is involved in the glucose deprivation-induced growth suppression of NSCs.** A, C17.2-NSCs were pretreated with Compound C (5 μM) or vehicle (DMSO) for 1 h. Cells were then incubated for 24 h with normal medium (25 mM glucose) or glucose-deprivation (0 mM glucose) in the presence of inhibitors, then an MTT incorporation assay was performed. The data are presented as the mean ± S.E. (n = 3) from three separate experiments. Bars represent the means ± S.D. of three different experiments. *, p < 0.05 as compared with glucose deprivation treated-pCAGGS-IRES-EGFP-transfected cells. ***, p < 0.001 as compared with normal medium (25 mM glucose) or glucose-deprivation (0 mM glucose) without inhibitor. **, p < 0.01 as compared with glucose deprivation treated-pCAGGS-IRES-EGFP-transfected cells. B, proliferation analysis of DN-AMPK-transfected C17.2 exposed to glucose deprivation or normal growth medium for 24 h using empty pCAGGS-IRES-EGFP vector as a control. C, cell viability was determined by MTT assay where absorbance at 570 nm is directly proportional to viable cell number. Bars represent the means ± S.D. of three different experiments. *, p < 0.05 as compared with glucose deprivation treated-pCAGGS-IRES-EGFP-transfected cells. Bars represent the means ± S.D. of three different experiments.

C, immunoblot analysis of phospho-Rb and the protein level of cyclin D in the cell lysate of DN-AMPK-transfected C17.2 after glucose deprivation for 24 h. The overexpression of DN-AMPK and the decrease of endogenous AMPK activity by dominant suppression were confirmed by blotting with anti-pan-AMPK antibody and anti-phospho-ACC antibody in DN-AMPK(C17.2) exposed to glucose deprivation for 24 h. Western blot results revealed that transfected DN-AMPK dramatically decreased the AMPK activity and also abolished the glucose deprivation-mediated down-regulation of cyclin D and phospho-Rb. The blots are representative of three individual experiments.

is in agreement with a previous report that NSC will differentiate after growth factor withdrawal, whereas at the same time FACS analysis demonstrated that they arrested in G_{0}/G_{1} phase (33, 34).

Interestingly, although we have not found apoptosis in AICAR-treated NSCs, as judged by no obvious DNA condensation or apparent PARP cleavage products (85-kDa cleaved fragment), we detected a slight increase (10-fold) in caspase-3 activity. Recently, Fernando et al. (35) demonstrated that NSC differentiation is dependent upon endogenous caspase-3 activity (7-fold increase reported), a conclusion supported by their other report which suggested that caspase-3 activity is also required for skeletal muscle differentiation (9.5-fold increase reported) (36). Their results suggest that non-apoptotic caspase-3 activity probably leads to the activation of cellular cytoskeletal remodeling signaling proteins such as p21/Cdc42/Rac-activated kinase 1 (PAK1) and apoptosis signal-regulating kinase 1 (ASK1), but not the cleavage of PARP and DNA fragmentation, in differentiating neurospheres (35, 37). This may also occur in our AICAR-induced differentiation model. Therefore, the activation of caspase-3 may represent an early step in stem cell differentiation.

Although the 86-kDa PARP cleavage fragment, typically associated with apoptosis caused by caspase-3, was not apparent after 48 h of AICAR treatment, a dramatic decrease in soluble full-length PARP protein was detected. We hypothesized that AICAR probably down-regulates the expression of PARP protein. PARP catalyzes poly-(ADP-ribosylation), a reversible post-translational protein modification implicated in the regulation of a number of biological functions. Although its role in the DNA damage response associated with cell survival and death has been recognized, recent work indicates that poly(ADP-ribosylation) is necessary for the transition from G_{0} phase to G_{1} phase, and the inhibition of PARP activity interferes with the up-regulation of immediate-early genes such as c-Fos and c-Myc (38). Other reports also indicate the involvement of PARP in cell differentiation and underscore an important function of PARP in connecting cell cycle progression and control of differentiation. De Blasio et al. (39) showed that inhibition of PARP allows human osteosarcoma MG-63 cells to restrict growth by G_{1} arrest accompanied with down-regulation of gene products required for proliferation (cyclin D, c-Jun, and Id2) and enter differentiation. Harnacke et al. (40) also reported that down-modulation of PARP restores transcriptional responsiveness for differentiation and cell cycle arrest. These results are similar to our observations of AICAR-induced PARP down-regulation, growth progression, and differentiation in NSCs, the link between which needs further investigation.

Together with a recent study of AICAR-induced astrogliogenesis, all the above results suggest that AICAR is a dual-function molecule inducing NSC G_{0}/G_{1} arrest and astroglial differentiation. Current observations from other research groups have also highlighted the existence of mechanisms coupling cell cycle exit and differentiation as well as functional cross-talk between intrinsic factors controlling these two mechanisms (41–44). PARP down-regulation may be an interesting mechanism for us to further investigate the connection between proliferation inhibition and differentiation induced in NSC by AICAR. Our observation that with regard to the phosphorylation state of AICAR inside the cell, ZMP is essential for not only the anti-proliferative effect (Fig. 3A) but also the astrogligenic...
AMPK Activation Inhibits Proliferation of Neural Stem Cells

effect (data not shown) demonstrates that ZMP is probably the key molecule coupling cell cycle exit with astroglial differentiation. The details of how ZMP couples these two mechanisms are still unclear and will be further investigated. It is also of interest for us to investigate the effect of AMP, the physiological analogue of ZMP, on the proliferation and differentiation of NSCs.

AICAR was the earliest known AMPK activator, and most of its effects have been shown to be the result of AMPK activation. However, in our recent study, the effect of AICAR on astroglial differentiation of NSCs was independent of AMPK but occurred via activating the JAK/STAT3 pathway. In this report we further investigate the role of AMPK and the JAK-STAT3 pathway in the anti-proliferative effect of AICAR on NSCs. Several lines of evidence point strongly to an important role of AMPK in this effect. First, iodotubericidin, which inhibits adenosine kinase by blocking the conversion of AICAR to its active form ZMP inside the cell, could completely block the anti-proliferative effect of AICAR, indicating that ZMP formation is required for this suppression process (Fig. 3A). Second, treatment with the AMPK inhibitor Compound C could also partially but significantly block the inhibitory effect of AICAR on NSC proliferation (Fig. 3B). Furthermore, dominant-negative mutants of AMPK could partially but significantly (***, p < 0.001, n = 3) block AICAR-induced cell cycle arrest (Fig. 3D), consistent with their blockade of the AICAR-mediated down-regulation of cyclin D and phospho-Rb (Fig. 4C). The JAK/STAT3 pathway seems not to be essential in this anti-proliferative effect of AICAR, as CNTF has no anti-proliferative effect on NSCs (Fig. 4, A–C), and the JAK-specific inhibitor fails to reverse the anti-proliferative effect of AICAR (Fig. 4D).

Because its discovery, investigations into the regulation and the effects of AMPK have expanded very rapidly (for review, see Ref. 45). Among these, research on the regulation of cellular proliferation by AMPK is becoming one of the most important areas. AMPK activation by AICAR has been reported to inhibit the proliferation of tumor cell lines including PC-3, C6, K-562, MCF-7, U87MG, and CEM by arresting the cell cycle in S phase via inhibiting the phosphatidylinositol 3-kinase/AKT/mTOR pathway and up-regulation of the p53/p21 axis (46). Gwinn et al. (47) recently reported that AMPK could directly phosphorylate the mTOR binding partner raptor, which is required for the inhibition of mTORC1 and cell cycle arrest induced by energy stress. In vascular smooth muscle cells, AMPK was reported to be able to induce cell cycle arrest in G1/G0 phase but not apoptosis via p27/p53 up-regulation and phospho-Rb down-regulation (48). In neuronal cell types, AMPK activation by AICAR has been reported to have a protective effect against apoptosis induced in astrocytes by fatty acids via Raf/extracellular signal-regulated kinase pathway inhibition (25) and by glucose deprivation in hippocampal neurons (26). Our data reported here suggest that in NSC, AICAR-induced AMPK activation suppresses proliferation and induces cell cycle arrest in G1/G0, probably associated with down-regulation of the cyclin D/phospho-Rb axis. Indeed, we measured the level of p53 and p21 proteins after AICAR treatment, but unfortunately, very weak bands were detected in both C17.2 and primary NSCs, consistent with a previous report that p21 and p27 are not enriched in NSCs (31). It was also reported that under physiological conditions, the level of p53 detected in the subventricular zone was very low (49). All of these conclusions are consistent with the rapid proliferation and self-renewal properties of NSCs. Similarly, microarray analysis of human embryonic stem cells showed that p53 and several genes that are known to cooperate with p53 in the regulation of cell cycle, including p16, p19, and p21, are not expressed or are expressed at very low levels (50, 51). We, thus, presumed that the difference between neural stem and other cell types in the expression profile of cell cycle regulators is responsible for the quite different results of AMPK activation. As to how AMPK activation down-regulates the cyclin D-Rb axis, we speculate that it may be through the mTOR pathway because we detected a decrease of phospho (Ser-2448)-mTOR after AICAR treatment (data not shown).

As an intracellular energy sensor, AMPK is activated under conditions that deplete cellular ATP and elevate AMP levels, such as glucose deprivation (52, 53), hypoxia (54), oxidative stress (55), and nitric oxide (56). Because AMPK activation induced by AICAR suppresses growth of NSCs, it was of interest for us to investigate the physiological role of AMPK in NSC survival under conditions of cellular stress induced by depletion of energy such as glucose deprivation. Our data indicate that glucose deprivation can also activate the AMPK pathway (Fig. 5A), induce G0/G1 arrest, but not apoptosis (Fig. 6, A and B), and suppress the proliferation of NSCs (Fig. 5, B and C) and that this effect is associated with decreased phospho-Rb and cyclin D protein (Fig. 6C). Furthermore, the AMPK inhibitor Compound C and overexpression of dominant-negative mutants of AMPK in C17.2 NSC could partially block the glucose deprivation-mediated down-regulation of cyclin D (Fig. 7C) and reverse the suppression of proliferation (Fig. 7, A and B), indicating that AMPK probably plays an important role in the glucose deprivation-induced cell cycle arrest and growth inhibition. Noticing a previous report that AMPK is expressed throughout the development of brain and serves as a mechanism for promoting neuron survival under conditions of reduced energy availability (47), we suggest that AMPK is probably involved in the response of NSC to microenvironmental energy stresses such as glucose starvation by limiting cell growth not only in the fast developmental expansion stage but also in the adult quiescent stage.

In summary, this study has revealed that both AICAR and glucose deprivation induce a G0/G1 arrest in NSCs and down-regulate the cyclin D/Rb axis via AMP-activated protein kinase but not the JAK/STAT3 pathway. This finding suggests that AMPK, a conserved cellular energy sensor, may play an important role in regulating the ability of NSC to adapt to an energy-limited microenvironment by limiting cell growth to reduce energy consumption. This finding will further our understanding of the mechanism of response of NSC to environmental stress and also expand our understanding of the physiological effects of AMPK.

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