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AMP-Activated Protein Kinase Is Essential for Survival in Chronic Hypoxia

Darrell R. Borger¹, L. Cristina Gavrilescu², Maria C. Bucur², Mircea Ivan^{2,3}, and James A. DeCaprio^{1,4}

¹Dana-Farber Cancer Institute, Department of Medical Oncology; Harvard Medical School, Boston, Massachusetts 02115

²Tufts-New England Medical Center, Molecular Oncology Research Institute, Boston, Massachusetts 02111

Abstract

This study was undertaken to interrogate cancer cell survival during long-term hypoxic stress. Two systems with relevance to carcinogenesis were employed: fully transformed BJ cells, and a renal carcinoma cell line (786-0). The dynamic of AMPK activity was consistent with a prosurvival role during chronic hypoxia. This was further supported by the effects of AMPK agonists and antagonists (AICAR and Compound C). Expression of a dominant-negative AMPK alpha resulted in decreased ATP level, and significantly compromised survival in hypoxia. Dose dependent pro-survival effects of rapamycin were consistent with mTOR inhibition being critical downstream of AMPK in persistent low oxygen.

Keywords

Hypoxia; AMPK; mTOR; rapamycin; Apoptosis; Cancer

INTRODUCTION

Identifying molecular events that enable cancer cells to survive and proliferate in a low oxygen microenvironment represents an area with profound implications for therapeutic developments in oncology [1]. Solid tumors contain regions of low oxygen tension, forcing neoplastic cells to undergo complex metabolic adaptations. It is conceivable that such processes can be incompletely understood by performing experiments that last less than 24 hours, which is the case for most published studies. The 5'AMP-dependent protein kinase (AMPK) is an energy sensor [2] containing a catalytic α subunit and regulatory β and γ subunits. AMPK is activated mainly by stresses that increase the AMP:ATP ratio, such as nutrient and glucose deprivation, exercise, ischemia [3,4,5] and hypoxia [6,7,8]. Increased AMPK activity has been identified in viable hypoxic regions of xenografts, while AMPK-defective cells were compromised in their ability to form tumors [9].

³Corresponding author: Mailing address: 750 Washington Street, Box 5609, Boston, MA 02111. Phone: (617) 636-7514. Fax: (617) 636-6127. E-mail: mivan@tuftsmedicalcenter.org.

⁴Corresponding author: Mailing address: Dana-Farber Cancer Institute, Mayer Building 440, 44 Binney Street, Boston, MA 02115. Phone: (617) 632-3825. Fax: (617) 582-8601. Email: james_decaprio@dfci.harvard.edu

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Activation of AMPK has been shown to enhance the function of the Tuberous Sclerosis Complex 2 (TSC2) and consequently inhibit the activity of the mTOR kinase (mammalian target of rapamycin) [10,11]. Although down-regulation of mTOR through TSC1-TSC2 in hypoxia has been demonstrated previously [12,13], dilemmas persist with regards to the relevant activator(s). While the published data are supportive of a critical role of AMPK in the survival of tumor cells in prolonged hypoxia, a direct experimental evidence is still missing.

MATERIALS AND METHODS

Plasmids

The retroviral construct pLB(N)CX AMPK α 2 D157A was generated by subcloning the N-terminal rat AMPK α 2 D157A (AMPK-DN) (gift from Laurie Goodyear, Joslin Diabetes Center, Boston) using 5'-*Sall* and 3'-*HindIII* restriction digestion. The resulting fragment was 5'-end-filled and cloned into the *HpaI* site of pLB(N)CX.

Cells and cell cultures

Human BJ fibroblast cells transformed through expression of *hTERT*, SV40 Large T antigen (LT), SV40 small T antigen (ST) and H-Ras G12V, were a gift from William Hahn (Dana-Farber Cancer Institute) [14]. A polyclonal Transformed BJ cell population stably expressing AMPK-DN was produced using amphotropic retroviruses derived from the Phoenix-Ampho retroviral producer line (from Garry Nolan, Stanford University). Retroviral vectors expressing the drug resistance gene were used to produce matched control cell lines. Stable polyclonals were subsequently selected using 5 μ g/ml blasticidin S. 786-0 renal carcinoma cells reconstituted with wild-type VHL and the vector-only controls were a gift from William Kaelin (Dana-Farber Cancer Institute). Cells were cultured in DMEM (Cellgro) buffered with 25 mM HEPES (pH 7.4), containing 4.5 g/L D-glucose, 584 mg/L L-glutamine, 110 mg/L sodium pyruvate and supplemented with 10% Fetal Bovine Serum (HyClone), 100 U penicillin per ml and 100 μ g streptomycin per ml (Gibco).

Hypoxia exposure and cell survival monitoring

Cells were propagated in an *InVivo200* workstation (Ruskin Technology Ltd, UK) at 37°C and exposed to 0.2% O₂, 5% CO₂ for a duration of 1 to 16 days. Medium was changed with pre-equilibration in hypoxia every 48 hours. Cells were plated at 50,000 (low-density) or 800,000 (high-density) per well in 6-well plates and growth curves were generated by cell counting with trypan blue exclusion.

Chemicals

5-Aminoimidazole-4-carboxamide 1- β -D-ribofuranoside (AICAR) (Sigma) compound C (Calbiochem), rapamycin (Sigma-Aldrich).

Antibodies

For western analyses, we used the commercial antibodies against the following proteins: phospho-AMPK α Thr172 (40H9); AMPK α ; acetyl CoA carboxylase; phospho-acetyl-CoA carboxylase Ser79; p70S6 kinase; phospho-p70 S6 Kinase Thr389 (108D2); cleaved caspase-3 Asp175 and lamin A/C (all from Cell Signaling Technology); HIF-1 α (NB 100–105) and HIF-2 α (NB 100–132) (from Novus Biologicals); Vinculin (Sigma-Aldrich); HA epitope (HA. 11) (Covance).

Western blotting

Cells were washed with PBS equilibrated under hypoxic conditions and lysed for 30 minutes in ice-cold NETN (20mM Tris-HCl [pH 8.0], 100mM NaCl, 1mM EDTA [pH 8.0], 0.5%

Nonidet P-40), supplemented with 1× Calbiochem Protease Set I and 1× Sigma-Aldrich Phosphatase Inhibitor Cocktail I. Extracts were cleared by centrifugation and protein concentration was quantified by the Bradford Assay. Lysates were denatured by boiling in loading buffer, separated using SDS-PAGE and transferred to nitrocellulose. Membranes were blocked in 5% nonfat dry milk, TBS-T (10mM Tris-HCl [pH 8.0], 150mM NaCl, 0.1% Tween 20) for 1 hour, then incubated overnight at 4°C in primary antibody diluted in 1% bovine serum albumin, followed by horseradish peroxidase-conjugated goat antibody (Pierce) at a 1:5,000 dilution. Visualization was performed using SuperSignal West Pico solution (Pierce) and chemiluminescent signals quantified using the Fujifilm LAS-3000 analyzer.

ATP quantification

Was performed using the ATP Bioluminescent Somatic Cell Assay (Sigma-Aldrich). Briefly, 2×10^5 cells/well were plated in triplicates in 6-well plates, in presence or absence of 250 μ M or 1mM AICAR, and submitted to normoxia or hypoxia for 3 days. Cells were then trypsinized, washed in PBS, incubated in ATP-releasing agent, followed by exposure to luciferin/luciferase. Light emission (RLU) was normalized to the number of cells in each sample.

RESULTS and DISCUSSIONS

Cell Survival During Extended Hypoxia and Dynamic of AMPK Activity

We first examined the response to hypoxia of human BJ fibroblasts transformed by sequential introduction of hTERT telomerase subunit, Large T and small T SV40 antigen, and the active H-Ras G12V mutant, set of genes known to render human cells tumorigenic [14]. However, transformed BJ cells can be considered “hypoxia naïve” (unlike established cancer cell lines), as they had not undergone prior selection for survival in a tumor microenvironment.

For the described experiments, cells were exposed to 0.2% O₂ for up to 16 days. This oxygen concentration, while extremely low, is within the range identified in solid tumors. Since acidification of the medium could complicate interpretation of the experiments, we used medium with increased buffering capacity, as previously described [15], which prevented the pH from falling below 7.1 during hypoxia exposure. Transformed BJ cells plated at low-density (~5–10% confluence) reproducibly proliferated during the first 6 days with little sign death until reaching confluence, followed by a phase dominated by apoptosis (day 6–8) as indicated by activation of caspase-3 (Fig. 1A). The surviving cells continued to proliferate, reaching again confluence around day 10, being mostly viable until the end of the experiment (day 16). HIF-1 α and HIF-2 α as assayed by western blotting, were elevated during the first 4 days of hypoxia, but were dramatically reduced during the more advanced phases (8 to 16 days) (Supplementary Fig. 1). When BJ cells were incubated in hypoxia at initial high density (see Methods), proliferation occurred during the first 2 days, followed by dramatic cell death between day 2 and 6 (Fig. 4).

In addition to the transformed BJ cells, we also investigated the behavior in chronic hypoxia of 786-0 renal carcinoma cells, known to exhibit homozygous inactivation of the von Hippel-Lindau (VHL) tumor suppressor gene. Loss of VHL results in a fundamental defect in oxygen sensing: normoxic stabilization of HIF alpha subunits [16,17]. We examined 786-0 cells reconstituted with the wild-type VHL gene (786-0VHL) versus cells expressing the corresponding empty vector (786-0Vect). In keeping with previous studies, 786-0VHL expressed low basal HIF-2 α levels, which transiently increased during acute hypoxia, and returned to almost normal levels in chronic hypoxia. In contrast, 786-0Vect expressed high HIF-2 α levels in normoxia and throughout the hypoxic exposure (Fig 1C). However, when plated at initial high density in hypoxia, both 786-0Vect and 786-0VHL survived and proliferated only during the first 2 to 4 days (Fig. 1D) followed by progressive cell death over

the course of 4 to 8 days. When plated at low density, 786-0 cells (with or without VHL) behaved similarly to the transformed BJ cells (see above). This result indicates that HIF- α status is not critical for survival in long-term hypoxia, at least in this cell type. One possible corollary of this experiment is that HIF overexpression could be paramount importance for renal cell carcinomas in vivo, as these types of tumors could be particularly sensitive to the lack of oxygen, and therefore require rapid development of dense vasculature.

We subsequently examined the dynamic of AMPK activity in chronic hypoxia in both transformed BJ cells and 786-O- derived clones, by monitoring the level of phospho-AMPK α Thr172, which mirrors the level of kinase activity [18]. In transformed BJ cells, Thr172 phosphorylation was not detectable in normoxia or early in hypoxia (1 day), but was high after 8 days, and even higher at 16 days (despite declining AMPK α levels) (Fig. 1A). Interestingly, induction of AMPK activity corresponded with declining HIF levels (Supplementary Fig. 1).

A contrasting dynamic of AMPK activity was observed in 786-0 cells, suggesting that its induction during hypoxia is not universal. Indeed, western analysis revealed that phospho-AMPK α Thr172 was high in normoxia and after 1 day of hypoxia, but dramatically decreased after 4 days. Importantly, phospho-AMPK dynamic was similar in 786-0VHL and 786-0Vect cells, indicating that this response was independent of HIF status (Fig. 1C). Glucose concentration monitoring at various points during the experiments described above indicated very little if any decrease (not shown), arguing that glucose depletion was unlikely to contribute to cell death in the system.

AMPK Activity Promotes Survival during Prolonged Hypoxia

To determine whether AMPK activity promotes survival in response to prolonged hypoxia, we performed both chemical and genetic manipulation of this pathway, in transformed BJ and 786-0 cells. For the chemical approach, cells were exposed to hypoxia in the presence of 1mM 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR), a widely used activator of AMPK [19], or compound C, a small molecule inhibitor of the kinase [20]. The corresponding solvents were used as controls.

As discussed above, transformed BJ cells incubated high-density in hypoxia proliferated during the first 2 days followed by progressive death thereafter (Fig. 2A). Treatment with AICAR, remarkably, almost completely blocked the death phase (the initial increase in cell number during the first 2 days of hypoxia, was attenuated, but not completely blocked). Conversely, the hypoxic death phase was dramatically accelerated by treatment with the AMPK inhibitor compound C. At the dose tested, Compound C did not display cytotoxic effects under standard normoxic culture conditions. Light microscopy confirmed the relative effects of AICAR or compound C treatment on survival after 6 days of continuous hypoxia (Fig. 2A). In parallel, we confirmed that treatment with these chemicals had the expected effect on AMPK activity. Thus, AICAR enhanced induction of pThr172, while compound C completely blocked induction over 4 days of hypoxic exposure, compared to untreated cells (Fig. 2B). Moreover, we examined phosphorylation of acetyl-CoA carboxylase (ACC) on the Ser79 residue, a known target of AMPK [21,22]. Phospho-ACC followed the trend of phospho-AMPK α , as its level was increased by AICAR and decreased by compound C treatment (Fig. 2B).

Finally, the decrease in cell number in compound C-treated samples in hypoxia was accompanied by high levels of cleaved caspase-3, supporting the impact of this chemical on apoptosis, conceivable via blocking AMPK activity. The untreated cells also exhibited increased levels of Caspase 3 at day 4, although the levels were much lower than in the compound C-treated counterparts. At longer exposure, the cleaved Caspase signal became well

visible in untreated cells, while in the AICAR-treated samples it was still undetectable (not shown).

A similar experiment was performed in 786-0 cells. While these cells underwent substantial cell death over a course of 8 days of continuous hypoxia, AICAR treatment led to a dose-dependent increase in hypoxic survival and induction of pThr172. (Supplementary Fig. 2).

Given the known limitations of small molecule agonists, we further interrogated the role of AMPK using a dominant-negative AMPK α 2 D157A mutant (AMPK-DN) [23]. The corresponding kinase-dead protein product sequesters the regulatory subunits, leading to the destabilization of the endogenous alpha component. Stable expression of the AMPK-DN led to a reduction in pThr 172 AMPK α and pACC compared to the vector-only control. Additionally, expression of the AMPK-DN blunted the increase in pThr172 and pACC Ser79 in response to AICAR (Fig. 3A). The effect was not dramatic, but reproducible (2–3 fold when quantified by densitometry). Additionally, expression of AMPK-DN was associated with dramatic cell death between day 4 to 6 of sustained hypoxia. In sharp contrast, the vector-only controls grew to confluence with no sign of death over the same period of time (Fig. 3B). There was no noticeable difference between the survival of AMPK-DN- expressing and controls during the first 2 days of hypoxia, arguing for the need of extended exposure when interrogating the role of energy sensors in hypoxia. Notably, under standard normoxic conditions AMPK-DN did not alter the population dynamic compared to vector-only controls (Fig. 3C).

Since energy generation in hypoxia is viewed as a major determinant for adaptation and survival [24], we interrogated the ATP dynamic in hypoxia and normoxia, in response to AICAR treatment or to AMPK DN expression. Interestingly, both 786-0 and BJ cells in chronic hypoxia are capable to generate ATP at comparable, if not higher levels compared to normoxia. However, AICAR treatment had a significantly elevated ATP levels, both in normoxia and hypoxia, consistent with its prosurvival effects. The opposite was seen in response to AMPK-DN transduction, which blunted ATP level in normoxia, hypoxia and in response to AICAR treatment (Supplementary Fig. 3).

mTOR Inhibition Has Prosurvival Effect in Hypoxia

We went on to test a downstream AMPK effector pathway that could account, at least in part, for the protective effect. Activation of AMPK has been shown to enhance the function of the Tuberous Sclerosis 2 complex (TSC2) and consequently inhibit mTOR (mammalian target of rapamycin) [10,11]. Reduced mTOR activity in hypoxia is predicted to have a significant impact in restoring the energy balance, as it leads to decreased protein synthesis. Although down-regulation of mTOR function via TSC1-TSC2 in response to hypoxic stress has been demonstrated previously, the studies have been performed either on cells defective in TSC2 signaling, or have involved additional stresses such as glucose deprivation [10,11,12,13].

Transformed BJ cells were plated at high-density and exposed to 4 days of hypoxia in the presence of different doses of rapamycin, a specific inhibitor of mTOR (in parallel with AICAR treatment). During the initial 2 days of hypoxic exposure, treated and non-treated cells survived and proliferated similarly. Beyond 2 days, rapamycin enabled cell growth to overconfluency in a dose-dependent manner, in sharp contrast to the non treated cells (Fig. 4). Additionally, in the experiments presented in Fig. 2 inhibition of AMPK activity by compound C was associated with detectable levels of mTOR target phospho-S6K Thr389 [25], in keeping with the idea that preservation of mTOR activity in deep chronic hypoxia has a robust apoptotic effect, at least in some cell types. These data are consistent with the data reported by Hamanaka et al [26]. We do not argue that mTOR repression is the only component downstream of AMPK important for cell survival in deep extended hypoxia, nor that the only reason for its inhibition is AMPK activation. Indeed, compound C eliminated any detectable p-Thr172 during hypoxia,

but only partially reversed the decrease in S6K Thr389 phosphorylation, consistent with mTOR receiving additional, AMPK-independent, inhibitory signals during oxygen deprivation.

It has been proposed that hypoxic REDD1 induction, rather than AMPK activation, is the critical event for mTOR inhibition [13]. Our data do not rule out a role for REDD1, which may well cooperate with AMPK for an inhibitory effect on mTOR. Another study demonstrated that oncogenic mutations in the PTEN/TSC2 pathway contribute to mTOR activity and increase protein translation under hypoxic condition [27]. However, we do not regard the latter as necessarily in contradiction with our results, as the selection pressure for a certain level of protein translation rate could vary with the depth and duration of hypoxia, as well as with the cell type in question.

From the standpoint of potential clinical implications, the efficacy of rapamycin-like drugs could be negatively affected by hypoxia, at least for some tumor types. Interestingly, the main application of the rapamycin analog temsirolimus is for the treatment of clear cell renal carcinomas [28], a highly vascularized (and conceivably more normoxic) tumor. In tumors with extensive/deep hypoxia, drugs from this family could paradoxically favor cell survival.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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FIGURE 1.

(A): Dynamic of phospho-AMPKα Thr172 (P-AMPKα) in transformed BJ cells exposed to prolonged hypoxic stress; AMPKα total amount of alpha subunit; Cleaved Caspase-3 western blotting monitors apoptotic cell death; Vinculin: loading control.

(B): Survival of transformed BJ cells plated at low-density in hypoxia. Cell numbers were normalized to day 0; measurement performed in triplicate.

(C): Levels of HIF-2α, reconstituted VHL (HA-tagged), total AMPKα and pThr172 (P-AMPKα), during hypoxic exposure in 786-O derived clones. Vinculin: loading control.

(D): Survival of 786-O derived clones cells in hypoxia.

FIGURE 2.

Pharmacological manipulation of AMPK activity affects survival during extended hypoxic exposure.

(A): Top: Total number of viable transformed BJ cells during treatment with 1mM AICAR, 10μM compound C, or vehicle, over 6 days of hypoxic exposure (initial high density; counting in triplicate normalized to day 0). **Bottom:** Light microscopy images representing the relative effects of AICAR or compound C treatment on survival after 6 days of continuous hypoxia.

(B): Biochemical effect of AICAR and compound C on AMPK and downstream targets after 4 days hypoxia. ACC: total levels of acetyl-CoA carboxylase; P-ACC: ACC phosphorylated on Ser79 (inactive); S6K: ribosomal protein S6 kinase 1; P-S6K: active S6K (phosphorylated on Thr389); Cleaved Caspase-3 as indicator of apoptotic cell death; Vinculin: loading control.

FIGURE 3. AMPK dominant-negative (AMPK-DN) inhibits survival during the chronic phases of hypoxia (d: days).
(A) Transduction of AMPK-DN interferes with endogenous AMPK activity (basal or induced), assessed through phospho-AMPK α Thr172 (P-AMPK α) and phospho-ACC Ser79 (P-ACC) immunoblots; expression of AMPK DN was put in evidence using the HA tag.
(B) Survival and proliferation of low-density transformed BJ cells stably expressing AMPK-DN or Vector control under normoxia.
(C) Same experiment performed under hypoxia: dramatic decrease in the viability of cell expressing AMPK-DN beyond two days hypoxia.

FIGURE 4.

Pharmacological inhibition of mTOR promotes survival in hypoxia. Shown is the experiment on transformed BJ cells plated at initial high density.

(A) Effects of treatment with 5nM rapamycin, 1mM AICAR, or DMSO control.

(B) Dose-dependent effect of rapamycin on cell survival, visible at low nanomolar doses.