Increased AMP:ATP ratio and AMP-Activated Kinase Activity During Cellular Senescence Linked to Reduced HuR Function

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Abbreviations: ActD, actinomycin D; AICAR, 5-amino-imidazole-4-carboxamide riboside; AMPK, AMP-activated protein kinase; Dex, dexamethasone; ELAV, embryonic lethal abnormal visual; UTR, untranslated region

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Cytoplasmic export of the RNA-binding protein HuR, a process that critically regulates its function, was recently shown to be inhibited by the AMP-activated kinase (AMPK). In the present investigation, treatment of human fibroblasts with AMPK activators such as AICAR, antimycin A, and sodium azide inhibited cell growth and lowered the expression of proliferative genes. As anticipated, AMPK activation also decreased both the cytoplasmic HuR levels and HuR’s association with target radiolabeled transcripts encoding such proliferative genes. HuR function was previously shown to be implicated in the maintenance of a ‘young cell’ phenotype in models of replicative cellular senescence. We therefore postulated that AMPK activation in human fibroblasts might contribute to the implementation of the senescence phenotype through mechanisms that included a reduction in HuR cytoplasmic presence. Indeed, AMP:ATP ratios were 2- to 3-fold higher in senescent fibroblasts compared with young fibroblasts. Accordingly, in vitro senescence was accompanied by a marked elevation in AMPK activity. Evidence that increased AMPK activity directly contributed to the implementation of the senescent phenotype was obtained through two experimental approaches. First, use of AMPK activators triggered senescence characteristics in fibroblasts, such as the acquisition of senescence-associated β-galactosidase (β-gal) activity and increased p16^INK4a expression. Second, infection of cells with an adenoviral vector that expresses active AMPK increased senescence-associated β-gal activity, while infection with an adenovirus that expresses dominant-negative AMPK decreased senescence-associated β-gal activity. Together, our results indicate that AMPK activation can cause premature fibroblast senescence through mechanisms that likely involve reduced HuR function.
INTRODUCTION

The AMP-activated kinase (AMPK), an enzyme that is believed to serve as a metabolic sensor or a ‘low fuel warning system’ of the cell (1), was discovered three decades ago. However, details of its molecular composition, regulation, and function are only now beginning to be elucidated. Structurally, mammalian AMPK is a heterotrimer of three subunits: one catalytic (α) and two regulatory (β and γ) subunits (2, 3). AMPK activity is ubiquitous, although different isoforms of AMPK subunits display tissue-specific distribution, as well as preferential subcellular localization. AMPK is activated directly by elevations in AMP and inhibited by high concentrations of ATP. Conditions that elevate the AMP:ATP ratio in cells, such as growth factor depletion, hypoglycemia, ischemia in heart muscle, exercise in skeletal muscle, as well as treatment with arsenite, azide, oxidative agents, and the pharmacological agent AICAR (which mimics the effect of AMP), can cause activation of AMPK (4, 5). In turn, AMPK inhibits biosynthetic pathways, thus conserving energy, while it activates catabolic pathways, thereby generating more ATP (reviewed in 4).

AMPK has been shown to influence gene expression in a variety of ways (1). AMPK-mediated transcriptional regulation has been demonstrated in yeast, where the AMPK homologue SNF1 is a pivotal regulator of glucose-related gene expression at times of low fuel availability (4, 6). The influence of AMPK on gene transcription in mammalian cells has also been documented. Recently, AMPK was shown to phosphorylate the transcriptional coactivator p300, thereby modulating its ability to interact with many nuclear receptors (7). AMPK has also been proposed to influence the levels of transcription factor forkhead FKHR (8) and to alter the transcription of various genes, including GLUT4 (9). In addition, AMPK was recently reported to have an inhibitory effect on protein synthesis, associated with decreased activation of the mammalian target of rapamycin (mTOR)
signal transduction pathway and its effectors (10). Finally, AMPK has been shown to influence mRNA turnover by inhibiting the cytoplasmic export of the RNA-binding protein HuR (11). Since HuR is predominantly (>90%) localized in the nucleus of unstimulated cells, it has been proposed that the mRNA-stabilizing influence of HuR requires its translocation to the cytoplasm (12-7). The AMPK-imposed inhibition of HuR transport to the cytoplasm blocks HuR’s ability to stabilize and enhance the expression of target mRNAs, including those that encode vascular endothelial growth factor (VEGF), p21, cyclin A, cyclin B1, c-fos, tumor necrosis factor-α and glut-1 (13, 17-20). These HuR target mRNAs share the presence of AU-rich elements (AREs) in their 3’ untranslated region (UTR), which modulate their half-life (21, 22). AREs in many labile mRNAs are also the targets of additional RNA-binding proteins (20, 23-29).

Among the cellular events that are influenced by HuR and its target mRNAs is the process of in vitro senescence. Using two different human fibroblast models of in vitro senescence, we recently reported the influence of the RNA-binding protein HuR in regulating the expression of several genes whose expression decreases during senescence (30). We demonstrated that HuR levels, HuR binding to target mRNAs, and the half-lives of such mRNAs were lower in senescent cells. Importantly, overexpression of HuR in senescent cells restored a ‘younger’ phenotype, while a reduction in HuR expression accentuated the senescent phenotype (30). These studies highlighted a critical role of mRNA turnover during the process of replicative senescence, and specifically implicated HuR in the regulation of such events. In the present study, we set out to test the hypothesis that AMPK activity might be elevated with senescence. Upon discovering that indeed AMP:ATP ratios are higher in senescent cells, we found that AMPK activity was accordingly elevated in senescent cells. Given the influence of AMPK on HuR function, we further assessed whether differences in AMPK function
could modulate the process of \textit{in vitro} senescence. Activation of AMPK led to an enhancement of the senescent phenotype associated with reduced cytoplasmic HuR levels, HuR binding to target mRNAs, and target mRNA half-life. By contrast, reduced AMPK function brought about a young, proliferative phenotype. Our findings strongly support a role for AMPK in the implementation of the senescence phenotype and suggest that AMPK-regulated HuR may be a critical factor in this process.
MATERIALS AND METHODS

**Cell culture, transfection, infection and assessment of senescence-associated β-galactosidase activity**

Human IDH4 fibroblasts were generously provided by J. W. Shay (31). Early-passage (~15-20 population doublings, pdl) and late-passage (50-53 pdl) human diploid IMR-90 fibroblasts, as well as early-passage (25-30 pdl) and late-passage (54-58 pdl) human diploid WI-38 fibroblasts were from Coriell Cell Repositories (Coriell Institute, Camden, NJ). All fibroblasts were cultured in Dulbecco’s modified essential medium (Gibco-BRL, Gaithersburg, MD) supplemented with 10% fetal bovine serum and antibiotics. Unless otherwise indicated, IDH4 cell culture medium was further supplemented with 1 µM dexamethasone (Dex) for constitutive expression of SV40 large T-antigen to suppress senescence and stimulate proliferation (31). To induce senescence of IDH4 cells, Dex was removed from the culture media, regular serum was replaced with charcoal-stripped serum, and cells were assessed at different times thereafter. Actinomycin D, dexamethasone, 5-amino-imidazole-4-carboxamide riboside (AICAR), 5’-AMP, antimycin A, and sodium azide were from Sigma (St Louis, MO).

Adenoviruses expressing either the control gene GFP (AdGFP), a dominant negative isoform of the α1 subunit of AMPK [Ad(DN)AMPK], or a constitutively active isoform of the AMPK α1 subunit [Ad(CA)AMPK] (32), were amplified and titered in 293 cells using standard methodologies. Infections were carried out in serum-free DMEM for 4 h. Infection efficiency was determined by infection with AdGFP at various plaque-forming units (PFU)/cell, and assessment of the percentage of GFP-expressing cells 48 h later. For >90% infection of IDH4 and IMR-90 cells, 300 PFU/cell was required.
Assessment of senescence-associated-β-galactosidase (β-gal) activity was carried out as previously described (30, 33). Briefly, cells were seeded in 30-mm dishes, cultured in media without Dex for different lengths of time and fixed with a 3% formaldehyde solution. Cells were then washed and incubated with senescence-associated β-gal staining solution (1 mg/ml X-gal, 40 mM citric acid/sodium phosphate buffer, pH 6.0, 5 mM ferrocyanide, 5 mM ferricyanide, 150 mM NaCl and 2 mM MgCl₂) for 12-18 h to visualize senescence-associated β-gal activity (33).

**Northern and Western blot analysis and subcellular fractionation**

Northern blot analysis was carried out as previously described (34). An oligomer complementary to 18S (34) was 3’ end-labeled using terminal transferase enzyme, while PCR-generated fragments of cyclin A, cyclin B1, and β-actin cDNAs were random primer-labeled using Klenow enzyme (30); all labeling reactions were carried out in the presence of [α-32P]dATP. Signals on Northern blots were visualized and quantitated with a PhosphorImager (Molecular Dynamics, Sunnyvale, Ca).

For western blotting, whole-cell (20 µg) or cytoplasmic (40 µg) lysates were prepared as previously described (17) and were size-fractionated by SDS-polyacrylamide gel electrophoresis and transferred onto PVDF membranes. Monoclonal antibodies were used to detect HuR (Molecular Probes, Eugene, OR), histone deacetylase 1 (HDAC 1), and β-Actin Santa Cruz Biotech. (Santa Cruz, CA). Following secondary antibody incubations, signals were visualized by enhanced chemiluminescence, quantitated by densitometry, and normalized against a loading control (β-Actin for cytoplasmic and whole-cell samples, histone deacetylase 1 for nuclear protein samples). Quantitative values are presented as ‘Percent of untreated’ or ‘Percent of Ad(GFP)’.
**Preparation of radiolabeled transcripts**

For *in vitro* synthesis of radiolabeled transcripts, RNA from IDH4 cells was reverse transcribed, and the cDNAs generated were used as templates in PCR reactions to amplify the 3’UTRs of cyclin A, cyclin B1, cyclin E and c-fos cDNAs, as described (17, 35). All 5’ oligonucleotides contained the T7 RNA polymerase promoter sequence CCAAGCTTCTAATACGACTCACTATAGGGAGA (T7). To prepare the cyclin A 3’UTR template, oligonucleotides (T7)CCAGAGACACTAAATCTGTAAC and GGTAACAAATTTCTGGTTTATTTC (region 1499-2718) were used. To prepare the cyclin B1 3’UTR template, oligonucleotides (T7)GTCAAGAACAAGTATGCCA and CTGAAGTGGGAGCGGAAAAG (region 1369-1702) were used. To prepare the cyclin E 3’UTR template, oligonucleotides (T7)CACAGAGCGGTAAAGCAG and GGATAGATATAGCAGCACTTAC (region 1169-1714) were used. PCR fragments served as templates for the synthesis of corresponding RNAs (36), which were used at a specific activity of 100,000 cpm/µl (2-10 fmoles/µl).

**RNA electrophoretic mobility shift (REMSA) and supershift assays**

Reaction mixtures (10 µl) containing 1 µg tRNA, 2-10 fmol RNA, and 5 µg protein in reaction buffer (15 mM Hepes, pH 7.9, 10 mM KCl, 10% glycerol, 0.2 mM DTT, 5 mM MgCl2) were incubated for 30 min at 25°C and digested with RNase T1 (500 units/reaction) for 15 min at 37°C. Complexes were resolved by electrophoresis through native gels (7% acrylamide in 0.25× TBE buffer). Gels were subsequently dried and radioactivity visualized using a PhosphorImager. For supershift analysis, 1 µg of antibody was incubated with lysates for 1 h on ice before addition of radiolabeled RNA; all subsequent steps were as described for REMSA. For supershifts, anti-HuR (Molecular Probes) and anti-p38 (Pharmingen, San Diego, CA) antibodies were used.
**AMPK assay and determination of AMP:ATP ratios**

AMPK was assayed as described (37). Briefly, AMPK was immunoprecipitated from 5 µg of cell lysate using 1 µg anti-α1 and 1 µg anti-α2 polyclonal antibodies in AMPK immunoprecipitation (IP) buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 50 mM NaF, 5 mM Na pyrophosphate, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 0.1 mM benzamidine, 0.1 mM PMSF, 5 µg/ml soybean trypsin inhibitor) for 2 h at 4°C. Immunocomplexes were washed with IP buffer plus 1 M NaCl, then with a buffer containing 62.5 mM Hepes, pH 7.0, 62.5 mM NaCl, 62.5 mM NaF, 6.25 mM Na pyrophosphate, 1.25 mM EDTA, 1.25 mM EGTA, 1 mM DTT, 1 mM benzamidine, 1 mM PMSF, 5 µg/ml soybean trypsin inhibitor. AMPK activity in immunocomplexes was determined by phosphorylation of peptide HMRSAMSGLHLVKRR [SAMS (37)] in reaction buffer (50 mM Hepes, pH 7.4, 1 mM DTT, 0.02% Brij-35, 0.25 mM SAMS, 0.25 mM AMP, 5 mM MgCl₂, 10 µCi [(\(-32\)P]ATP) for 10 min at 30°C. Assay mixtures were spotted onto P81 filter paper and rinsed in 1% (v/v) phosphoric acid with gentle stirring to remove free ATP. Phosphorylated substrate was measured by scintillation counting. Measurement of AMP and ATP was carried out as previously described (38).

**Immunofluorescence**

Proliferating IDH4 cells were seeded on coverslips and either left untreated or treated with AMPK activators. At the end of the treatment period, HuR was detected by immunofluorescence as previously described (11) using anti-HuR (Santa Cruz Biotech.). Nuclei were visualized using Hoechst 33342 (Molecular Probes). Signals were detected using an Axiovert 200M microscope (Zeiss; 63× lens) using separate channels for the analysis of phase contrast images, red fluorescence...
Results

Treatment with AMPK activators causes growth arrest and decreased expression of proliferative genes in human IDH4 fibroblasts

In the IDH4 human fibroblast cell model, developed by Shay and colleagues (31), constitutive, dexamethasone (Dex)-driven SV40 large T antigen expression allows IDH4 cells to suppress senescence and to continue to proliferate as young cells. Treatment of proliferating IDH4 cells with the AMPK activators AICAR, antimycin A and sodium azide caused upregulation of AMPK activity (Fig. 1A), cessation of cell growth (Fig. 1B) and a marked reduction in the expression levels of proliferative proteins such as Cyclin A and Cyclin B1 (Fig. 1C). These reductions in protein abundance were further assessed by monitoring mRNA levels. As seen in Fig. 2A, treatment with either AICAR, antimycin A or sodium azide led to a corresponding reduction in the levels of mRNAs encoding cyclin A and cyclin B1. Given our earlier findings that cyclin A and cyclin B1 mRNA turnover changed according to the cell’s proliferative status (35), we sought to determine if such gene expression differences were influenced by changes in the stability of the corresponding mRNAs. We used an actinomycin D-based approach to assess the relative mRNA half-lives following treatment with AMPK activators. As shown in Fig. 2B, IDH4 cells showed significant differences in the half-lives of mRNAs encoding cyclin A and cyclin B1. For these mRNAs, treatment with actinomycin D resulted in faster transcript loss in the populations treated with AMPK activators, revealing that their stability was lowered by the treatments. Such differences in mRNA stability were specifically seen for cyclin A mRNA and cyclin B1 mRNA, as the stability of other mRNAs studied, such as that encoding...
β-actin, was unchanged (Fig. 2B).

**Treatment of IDH4 cells with AMPK activators reduces cytoplasmic HuR levels and decreases binding of HuR to cyclin A, cyclin B1 and c-fos 3’UTRs**

Cyclin A and Cyclin B1 are encoded by mRNAs that are targets of HuR binding in proliferating cells (35). In order to assess whether the AMPK-triggered reduction in expression of cyclin A and cyclin B1 might be linked to the ability of HuR to bind to the 3’ untranslated regions (UTRs) of these transcripts, RNA electrophoretic mobility shift assays (REMSA) were carried out. IDH4 cells were either left untreated or treated with AMPK activators, whereupon cytoplasmic lysates were prepared and incubated with radiolabeled RNAs encompassing the 3’ UTRs of the cyclin A and cyclin B1 mRNAs, as described (30). REMSA analysis was also carried out using transcripts corresponding to the 3’UTR of c-fos, a proliferative gene whose mRNA was previously reported to be a target of HuR (30) (Fig. 3). Remarkably, proteins present in cytoplasmic lysates prepared from untreated IDH4 cells revealed much more extensive binding to radiolabeled transcripts than did proteins present in cytoplasmic lysates from IDH4 cells treated with AMPK activators. Evidence that HuR was part of the REMSA complexes, and that HuR abundance was indeed greater in the untreated populations, was revealed through use of an anti-HuR antibody to supershift the [protein-RNA] associations. HuR-containing supershifted complexes (arrowheads, Fig. 3) were most abundant when lysates from untreated populations were used and were markedly reduced following treatment with AMPK activators; the specificity of the assay was tested using antibodies directed to control proteins (such as p38MAPK, which lacks RNA-binding ability), which did not produce supershifts (Fig. 3). For each transcript and treatment group tested, nuclear lysates were tested similarly and revealed no change in binding patterns or signal intensities after treatment with AMPK activators (not shown), in
agreement with earlier findings (11). Additional control REMSA analysis using non-HuR target transcripts such as those corresponding to each coding region [previously shown and therefore not shown here (35)] and that encompassing the cyclin E 3’UTR (Fig. 3), served to further assess the specificity of these associations. Taken together, the reduced association of HuR with cyclin A and B1 3’UTR transcripts under conditions that cause destabilization of the encoded mRNAs is in keeping with HuR’s reported ability to stabilize and enhance the expression of mRNAs of such proliferative genes (30, 35).

Given our earlier observations in RKO colorectal carcinoma cells, where the cytoplasmic localization of HuR was regulated by AMPK (11), we set out to assess the influence of AMPK activators on the subcellular distribution of HuR in IDH4 cells. As previously seen in RKO cells (11), treatment of proliferating IDH4 cells with AMPK activators AICAR, antimycin A and sodium azide also led to a time-dependent reduction in cytoplasmic HuR (Fig. 4A). Also in keeping with our previous findings using RKO cells, AMPK activators affected the subcellular localization of HuR, but did not modify the total or nuclear levels of HuR, which remained unchanged throughout the time-period examined (Fig. 4A). Confirmation of these findings was obtained using immunofluorescence (Fig. 4B). While HuR was mostly nuclear in all treatment groups, as previously shown (11, 30), the cytoplasmic signal observed in control IDH4 cells (untreated), was further reduced in populations treated with either AICAR, Antimycin A, or Azide (Fig. 4B). Given the relatively low abundance of cytoplasmic HuR in untreated IDH4 cells, detection by immunofluorescence was poor and all subsequent assessments of HuR subcellular localization was carried out by western blotting, which allowed use of sufficient cytoplasmic material for reliable analysis and quantitation.

**AMPK activity increases during cellular senescence**
The observations described thus far indicate that AMPK activators were capable of reducing cytoplasmic HuR levels in IDH4 cells. Such reductions in HuR expression are reminiscent of those we previously observed in cellular models of in vitro senescence (30). We thus hypothesized that changes in AMPK activity occurring during in vitro cellular senescence may underlie the changes in HuR function described during this process (30). First, we examined AMPK activity during the process of in vitro senescence. To this end, we employed the IDH4 model described above and compared AMPK activity in young cells (cultured in Dex), with that in IDH4 cells induced to undergo replicative senescence by removing Dex (and thereby inhibiting large-T antigen expression (30)] from the culture medium. As shown, AMPK activity, which was measured as indicated in the Materials and Methods section, increased rapidly following removal of Dex to induce senescence, remaining elevated throughout the time-period studied (Fig. 5A). The cytoplasmic levels of HuR in IDH4 cells treated with AMPK activators were comparable to those seen in IDH4 cells rendered senescent by removing Dex for 5 d (Fig. 5B). We investigated the senescence-associated AMPK activation in two other cell systems of replicative senescence: human diploid fibroblasts IMR-90 and WI-38 that were cultured for either a low (~20-30) or a high (~50-58) number of passages. As shown in Fig. 5C, young, low-passage WI-38 and IMR-90 cells exhibited low AMPK activity, while senescent, late-passage WI-38 and IMR-90 cells displayed significantly elevated AMPK activity. As shown in Table 1, measurement of AMP and ATP levels in two in vitro senescence models revealed a 2- to 3-fold increase in AMP:ATP ratios in senescent fibroblasts (IDH4 cells cultured without Dex, late-passage WI-38 cells), than in young fibroblasts (proliferating IDH4 cells, early-passage WI-38 cells).

**Interventions to increase AMPK activity accelerate fibroblast senescence, while reduction of AMPK activity delays senescence**
In order to assess if upregulation of AMPK activity could influence the implementation of the senescent phenotype, young IDH4 and IMR-90 fibroblasts were treated with AMPK activators and phenotypic features of senescence examined. As shown in Fig. 6A, neutral, senescence-associated β-galactosidase (β-gal) (33) was detected in treated cultures, but was largely absent from untreated cells. Senescence-associated β-gal activity is widely used as a biomarker for replicative senescence, although the specific enzyme(s) involved remain poorly characterized (33). In addition, expression of p16^{INK4a}, an inhibitor of cyclin-dependent kinases whose expression and function are strongly linked to cellular senescence (39), was markedly increased in IDH4 cells treated with AMPK activators (Fig. 6B).

Further demonstration that AMPK contributed to modulating cellular senescence came from experiments using adenoviral vectors to ectopically express mutant forms of the AMPK α (catalytic) subunit: adenovirus Ad(CA)AMPK, carrying α1312 (a constitutively active α1 mutant), and adenovirus Ad(DN)AMPK, which carries a dominant negative mutant of α1 (32). When compared with infections using a control adenovirus which expresses the GFP protein (AdGFP), infection with Ad(CA)AMPK led to an approximately 2.7-fold increase in AMPK activity in IDH4 cells (Fig. 7A), and a comparable increase in IMR-90 cells (Fig. 8A), with >90% cells infected at 300 PFU/cell (not shown). Importantly, such intervention led to a marked decrease in cytoplasmic HuR in both IDH4 (Fig. 7B) and IMR-90 cells (Fig. 8B). Conversely, infection with Ad(DN)AMPK led to ~40% reduction in AMPK activity in IDH4 cells (Fig. 7A) and markedly elevated their cytoplasmic HuR abundance (Fig. 7B). Neither nuclear (not shown) nor total cellular HuR (Fig. 7B) were altered by the infections. Interestingly, senescence-associated β-gal activity was elevated in cells displaying increased AMPK function, as shown for IDH4 and IMR-90 cells (Figs. 7C and 8C), while reduced AMPK activity decreased the levels of senescence-associated β-gal in IDH4 (Fig. 7C); this intervention could not be
studied in IMR-90 cells, since its higher constitutive AMPK activity could not be substantially reduced by Ad(DN)AMPK. Taken together, our findings indicate that AMPK may play an important role during the establishment of cellular senescence and points to HuR as a likely mediator of gene expression changes observed during AMPK-triggered senescence.

**DISCUSSION**

The present investigation originated at the point of convergence of two earlier studies from our laboratory. In the first study, we identified AMPK as an enzyme involved in regulating the cytoplasmic localization of HuR, and consequently HuR function in stabilizing target mRNAs and increasing their expression (11). In the second, we used several human fibroblast models of *in vitro* cellular senescence to show that HuR abundance decreased as fibroblasts progressed towards senescence (30); in these models, we showed that reduced HuR levels effectively contributed to the implementation of senescent phenotype. Here, we set out to test the hypothesis that reductions in cytoplasmic HuR levels in human fibroblasts, brought about by enhancing AMPK activity, were capable of inducing a senescent phenotype. Indeed, interventions that induced AMPK activity, such as treatment with AMPK activators and infection with an adenovirus that expresses a constitutively active AMPK isoform, caused premature senescence in IDH4 and IMR-90 cells. Conversely, reductions in AMPK activity caused delays in the onset of cellular senescence.

However, it was somewhat unexpected to find that both AMP:ATP ratios, and consequently AMPK activity, were *naturally* augmented in senescent fibroblasts. To our knowledge, this constitutes the first report that AMP:ATP ratios and AMPK activity increase with *in vitro* senescence. While elucidation of the precise mechanisms responsible for the senescence-related increase in AMP:ATP ratios (Table 1) is of great interest, such analysis could be rather complex, given the many possible
means of altering the cellular concentration of ATP and AMP, as discussed below. AMPK function can also be regulated by factors other than AMP:ATP ratios. For instance, the AMPK αβγ heterotrimer composition may be influenced by \textit{in vitro} senescence; while no acute changes in expression levels of specific subunits \([\alpha1, \alpha2, \beta1, \beta2, \gamma1, \gamma2, \gamma3\) have been documented, different heterotrimers have been shown to display tissue-specific distribution, as well as restricted subcellular localization. In addition to the particular \(\alpha\) and \(\gamma\) isoforms present in the complex, the degree of AMPK activation can also depend on whether or not it is phosphorylated by its upstream kinase, AMPKK (for review, see reference 1).

Generally speaking, AMPK is activated by stress situations that increase the cellular AMP:ATP ratio. Physiological situations that cause a reduction in ATP availability, such as exercise in skeletal muscle, have been shown to induce AMPK function. Similarly, certain pathological conditions that cause cellular hypoxia, hypoglycemia, oxidative stress, heat shock and other stresses, also elevate AMPK function. In this regard, oxidative damage accumulates in cellular components during normal mammalian aging, thereby impairing the cell’s ability to adequately perform a variety of functions. Such impairment in cellular functions, which has been linked to the decreased ability of old cells to activate stress response pathways and their diminished proliferative ability (40), has also been associated with the overall age-related decline in expression of genes encoding mitochondrial and energy metabolism proteins (41-43). Such age-related deficits and damage to cellular macromolecules involved in energy production could therefore underlie the age-related lowered ATP production (43, 44), elevated AMP:ATP ratio (Table 1), and consequently increased AMPK function.

Previous studies demonstrated that activated AMPK was capable of causing HuR to be predominantly nuclear and thereby blocked HuR’s ability to stabilize target mRNAs (11). In the
present investigation, we sought to obtain more direct evidence that HuR contributed to the establishment of a senescent phenotype when AMPK was induced. We encountered technical limitations that precluded such an analysis. Indeed, transient transfection experiments to test if HuR overexpression could block AICAR-, antimycin A- or azide-triggered senescence were unsuccessful due to the toxicity of the combined treatment (lipofectamine plus the AMPK activator). Stable HuR overexpression was not attainable in human diploid fibroblasts, as previously explained (30). However, in light of HuR’s influence on cellular senescence (30), we propose that HuR directly participates in the implementation of AMPK-triggered cellular senescence.

In summary, we describe for the first time that replicative senescence is characterized by elevations in AMP:ATP ratios and AMPK activity. Moreover, modulation of AMPK activity via adenoviral vectors or chemicals was capable of altering the senescent phenotype. Such modulations of AMPK activity effectively altered the relative abundance of cytoplasmic HuR and consequently the expression of HuR-regulated proliferative genes. Although AMPK activity has not been previously studied in the context of cellular or organism aging, it has been proposed to contribute to inhibiting cell proliferation. The observation that active AMPK reduces the expression of cyclin A, cyclin B1 and c-fos (11) supports the notion that AMPK could contribute to both acute growth arrest and growth arrest associated with cellular senescence. Despite the controversy surrounding the link between human aging and in vitro cellular senescence, diminished proliferative capacity characterizes in vivo aging. The discovery of AMPK’s involvement in cellular senescence therefore warrants a careful look at AMPK in other models of aging.
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REFERENCES


FIGURE LEGENDS

Figure 1. AMPK activity in IDH4 cells treated with AMPK activators AICAR, antimycin A, and sodium azide. (A) Actively proliferating IDH4 cells were treated with either 2 mM AICAR, 5 µM antimycin A, or 2 mM sodium azide, for 5 h and AMPK activity was determined as explained in the Materials and Methods section. (B) Fifty thousand IDH4 cells per well were treated with either AICAR, antimycin A or sodium azide at the doses indicated above, and cell numbers were assessed every 12 h using a hemacytometer. Data represent the means and standard error of the means. (C) Western blot analysis to monitor the expression of Cyclin A and Cyclin B1 in whole-cell lysates (20 µg) prepared from IDH4 cells that were treated as described in the legend of panel A.

Figure 2. Effect of AMPK activators on the expression and half-life of mRNAs encoding Cyclin A and Cyclin B1. (A) Northern blot analysis to assess the abundance of mRNAs encoding Cyclin B1, Cyclin A, and β-Actin in IDH4 cells following treatment with 2 mM AICAR, 5 µM antimycin A, or 2 mM sodium azide, for 5 h. (B) Stabilities of mRNAs encoding Cyclin A, Cyclin B1, and β-Actin in IDH4 cells that were cultured in the presence of AMPK activators as indicated in the legend of panel A. Half-lives were assessed after adding 2 µg/ml actinomycin D during continuous presence of the AMPK activators, preparing RNA at the times indicated, measuring cyclin A, cyclin B1, and β-actin Northern blot signals, normalizing them to 18S rRNA, and plotting them on a logarithmic scale. Dashed horizontal lines, 50% of untreated. Data represent the means ±SEM of 3 independent experiments.
Figure 3. Effect of AMPK activators on the ability of HuR to bind target mRNAs encoding Cyclin A, Cyclin B1 and c-Fos. REMSA assays were performed using radiolabeled RNAs encoding the 3’UTRs of cyclin A, cyclin B1, c-fos and cyclin E (Materials and Methods) and proteins present in cytoplasmic lysates of IDH4 cells that had been treated with 2 mM AICAR, 5 µM antimycin A, or 2 mM sodium azide, for 5 h. The presence of HuR in [RNA·protein] complexes was assayed by monitoring the formation of supershifted bands in the presence of anti-HuR antibodies (+αHuR ab) or control antibodies (+αp38 ab). Arrowhead, supershifted complexes.

Figure 4. Effect of AMPK activators on the subcellular localization of HuR. (A) Western blot analysis of HuR levels in Cytoplasmic (40 µg), Nuclear (20 µg), and whole-cell (Total, 20 µg) lysates prepared from IDH4 cells that were treated for the indicated times with either 2 mM AICAR, 5 µM antimycin A, or 2 mM sodium azide. Hybridizations to detect either β-Actin (Cytoplasmic and Total lysates) or HDAC1 (Nuclear lysates) served to monitor differences in loading of samples. (B) Detection of HuR in proliferating IDH4 cells that were either left untreated or treated for 5 h with AICAR, antimycin A or sodium azide at the concentrations described in panel (A). Top, phase contrast images; middle, HuR immunofluorescence; bottom, Hoechst staining to visualize nuclei.

Figure 5. Kinetics of AMPK kinase activity in IDH4, WI-38 and IMR-90 human fibroblasts undergoing senescence. (A) Assessment of AMPK activity in IDH4 cells following removal of dexamethasone (Dex) for the times indicated. (B) Western blot analysis of cytoplasmic HuR (40-µg protein aliquots per lane) in proliferating IDH4 cells (0 d) that were either left untreated or treated with the indicated AMPK activators (2 mM AICAR, 5 µM antimycin A, or 2 mM sodium azide) for 5 h, and in IDH4 cells rendered senescent by removal of Dex for 5 d. (C) Assessment of AMPK activity in
WI-38 fibroblasts (left) and IMR-90 fibroblasts (right) at the indicated population doublings. AMPK assays were performed as described in the Materials and Methods section.

Table 1. Assessment of AMP:ATP ratios in cells undergoing senescence. AMP and ATP levels in young cultures (proliferating IDH4 cells, early-pdl WI-38 cells) or senescent cultures (IDH4 cells cultured without Dex, late-pdl WI-38 cells) were calculated (38) and expressed as AMP:ATP ratios. Representative values from two or more measurements are shown.

Figure 6. Effect of AMPK activators on the senescent phenotype of IDH4 and IMR-90 fibroblasts. (A) Following treatment of IDH4 or IMR-90 cells with AMPK activators AICAR (1 mM), antimycin A (1 µM), and sodium azide (1 mM), for 7 d or 3 d, respectively, senescence-associated β-gal activity was examined as described in Materials and Methods. (B) Western blot analysis to measure p16INK4a expression in IDH4 cells treated as explained in the legend of Fig. 1.

Figure 7. Effect of adenoviral infection to modulate AMPK activity on the senescent phenotype of IDH4 fibroblasts. (A) Assessment of AMPK activity in IDH4 cells 5 d after infection with 300 PFU/cell of a control adenovirus (AdGFP) or with adenoviruses that express either a constitutively active isoform [Ad(CA)AMPK] or a dominant negative isoform [Ad(DN)GFP] of the AMPK catalytic subunit. Data represent the means ±SEM of 3 independent experiments. (B) Five d after infection of IDH4 cells as described in the legend of Fig. 7A, HuR expression in cytoplasmic (Cytoplasmic) and whole-cell (Total) lysates was determined by western blot analysis. (C) Five d after infection, senescence-associated β-gal activity was assessed as described in the Materials and Methods section.
Figure 8. Effect of adenoviral infection to modulate AMPK activity on the senescent phenotype of IMR-90 fibroblasts. (A) Assessment of AMPK activity in IMR-90 cells 3 d after infection with 300 PFU/cell of a control adenovirus (AdGFP) or with an adenovirus that expresses a constitutively active isoform [Ad(CA)AMPK] of the AMPK catalytic subunit. Data represent the means ±SEM of 3 independent experiments. (B) Three d after infection of IMR-90 cells as described in the legend of Fig. 8A, HuR expression in cytoplasmic lysates was determined by western blot analysis. (C) Senescence-associated β-gal activity was assessed 3 d after infection of IMR-90 using 300 PFU/cell of a control adenovirus (AdGFP) or with adenoviruses that express a constitutively active isoform [Ad(CA)AMPK], as described in the Materials and Methods section.
Wang et al., AMPK-triggered senescence linked to reduced HuR function, page 28

**A**

AMPK kinase activity (Fold, relative to untreated)

- Untreated
- AICAR
- Antimycin A
- Azide

**B**

Cells (×10,000)

- Untreated
- AICAR
- Antimycin A
- Azide

**C**

- Cyclin A
  - 100 32 24 35 % of untreated
- Cyclin B1
  - 100 64 26 12 % of untreated
- β-Actin

**Wang et al., Fig. 1**
Wang et al., AMPK-triggered senescence linked to reduced HuR function, page 29

A

- cyclin A
- cyclin B1
- 18S

B

Percent remaining mRNA

Time in actinomycin D (h)

Wang et al., Fig. 2
Wang et al., Fig. 3

Cyclin B1 3'UTR Cyclin A 3'UTR

Cyclin B1 3'UTR Cyclin A 3'UTR

C-fos 3'UTR Cyclin E 3'UTR

Wang et al., AMPK-triggered senescence linked to reduced HuR function, page 30
### A

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#### Cytoplasmic

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#### Nuclear

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#### Total

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### B

#### Phase Contrast

- **untreated**: Untreated control
- **AICAR**: AICAR treatment
- **Antimycin A**: Antimycin A treatment
- **Azide**: Azide treatment
- **(Neg)**: Negative control

#### HuR

- **untreated**: Normal level of HuR expression
- **AICAR**: Reduced HuR expression
- **Antimycin A**: Further reduced HuR expression
- **Azide**: Minimal HuR expression

#### Hoechst

- **untreated**: Normal nuclear staining
- **AICAR**: Normal nuclear staining
- **Antimycin A**: Normal nuclear staining
- **Azide**: Normal nuclear staining

---

Wang et al., Fig. 4
Wang et al., AMPK-triggered senescence linked to reduced HuR function, page 32

A

AMPK kinase activity (Fold, relative to control)

0 1 3 5 7 9
Time after Dex removal (d)

Young Sen.

iDH4

B

HuR -

β-Actin -

Time after Dex removal (d)

0 0 0 0 5
untreated AICAR Antimycin A Azide untreated untreated AICAR Antimycin A Azide untreated

C

AMPK kinase activity (Fold, relative to control)

0 1 2 3
WI-38 IMR-90

27 58 21 53
Population Doubling

Wang et al., Fig. 5
<table>
<thead>
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<tr>
<td>IDH4 Old (day 5)</td>
<td>0.13</td>
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<tr>
<td>WI-38 Young (pdl 27)</td>
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<tr>
<td>WI-38 Old (pdl 58)</td>
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</tr>
</tbody>
</table>

Wang et al., Table 1
**A**

IDH4

untreated | AICAR | Antimycin A | Azide

IMR-90

untreated | AICAR | Antimycin A | Azide

**B**

un treated | AICAR | Antimycin A | Azide

- p16

- β-Actin

Wang et al., Fig. 6
Wang et al., AMPK-triggered senescence linked to reduced HuR function, page 35

Wang et al., Fig. 7

A

AMPK kinase activity

[Fold, relative to Ad(GFP)]

0

1

2

3

Ad(CA)AMPK

Ad(GFP)

Ad(DN)AMPK

B

HuR -

% of Ad(GFP)

100 18 186

β-Actin -

Cytoplasmic

Total

C

Ad(GFP)

Ad(CA)AMPK

Ad(DN)AMPK

Wang et al., Fig. 7
Wang et al., AMPK-triggered senescence linked to reduced HuR function, page 36

Wang et al., Fig. 8

A

AMPK kinase activity [Fold, relative to Ad(GFP)]

Ad(GFP) Ad(CA)AMPK

B

HuR -  β-Actin -

% of Ad(GFP)  100  36

Cytoplasmic

C

Ad(GFP) Ad(CA)AMPK
Increased AMP: ATP ratio and AMP-activated kinase activity during cellular senescence linked to reduced HuR function
Wengong Wang, Xiaoling Yang, Isabel Lopez de Silanes, David Carling and Myriam Gorospe

J. Biol. Chem. published online May 1, 2003

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