AMP-activated Protein Kinase (AMPK)–Induced Preconditioning in Primary Cortical Neurons Involves Activation of MCL-1

Abbreviated Title: AMPK Preconditioning and MCL-1

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Abbreviations: AICAR, 5-aminoimidazole-4-carboxamide riboside AMPK, AMP-activated protein kinase BCL-2, B-cell lymphoma 2 CCD, charge coupled device CNS, central nervous system DIV, days in vitro GLUT 3, glucose transporter 3 HEPES, N-[2-hydroxyethyl]piperazine-N’-[2-emanesulphonic acid] MCL-1, myeloid cell leukemia sequence 1 PBS, phosphate buffered saline qPCR, quantitative PCR shRNA, small hairpin RNA siRNA, small interfering RNA TMRM, tetramethylrhodamine methyl ester
Abstract

Neuronal preconditioning is a phenomenon where a previous exposure to a sub-lethal stress stimulus increases the resistance of neurons towards a second, normally lethal stress stimulus. Activation of the energy stress sensor, AMP-activated protein kinase (AMPK) has been shown to contribute to the protective effects of ischemic and mitochondrial uncoupling-induced preconditioning in neurons, however the molecular basis of AMPK-mediated preconditioning have been less well characterised. We investigated the effect of AMPK preconditioning using 5-aminoimidazole-4-carboxamide riboside (AICAR) in a model of NMDA-mediated excitotoxic injury in primary mouse cortical neurons. Activation of AMPK with low concentrations of AICAR (0.1 mM for 2 h) induced a transient increase in AMPK phosphorylation, protecting neurons against NMDA-induced excitotoxicity. Analyzing potential targets of AMPK activation, demonstrated a marked increase in mRNA expression and protein levels of the anti-apoptotic BCL-2 family protein MCL-1 in AICAR-preconditioned neurons. Interestingly, overexpression of MCL-1 protected neurons against NMDA-induced excitotoxicity while MCL-1 gene silencing abolished the effect of AICAR preconditioning. Monitored intracellular Ca^{2+} levels during NMDA excitation revealed that MCL-1 overexpressing neurons exhibited improved bioenergetics and markedly reduced Ca^{2+} elevations, suggesting a potential mechanism through which MCL-1 confers neuroprotection. This study identifies MCL-1 as a key effector of AMPK-induced preconditioning in neurons.

Key Words: Excitotoxicity, Glutamate, Ca^{2+} homeostasis, mitochondria, bioenergetics, BCL-2 family
Introduction

Glutamate receptor overactivation has been postulated to play a major role in neuronal loss associated with ischemic stroke, traumatic and epileptic brain injury (Choi and Rothman, 1990). Neuronal death induced by glutamate receptor overactivation is crucially dependent on the magnitude of ionic imbalance, mitochondrial dysfunction, oxidative stress, glucose availability and ATP depletion (Choi, 1987, Castilho et al., 1998, Delgado-Esteban et al., 2000). During excitotoxic injury neurons can undergo rapid necrosis, a PARP-dependent cell death pathway (parthanatos), or a more delayed form of caspase-independent, calpain-dependent apoptosis. Ultimately which cell death pathways are activated depends on intrinsic neuronal properties as well as intensity and duration of the excitotoxic insult (Lankiewicz et al., 2000, Ankarcrona et al., 1995, D'Orsi et al., 2012, Yu et al., 2002). Delayed, caspase-independent excitotoxic apoptosis differs from necrosis or parthanatos in that the initial ionic and energetic imbalance initially recovers. Nevertheless neurons die subsequently as a result of mitochondrial permeabilization, a process that is controlled by BCL-2 family proteins (Ward et al., 2007). BCL-2 family proteins either promote or inhibit apoptosis (Youle and Strasser, 2008). They are classified into pro-apoptotic activators (BH-3 only proteins such as BIM, PUMA, BID, BAD, NOXA), pro-apoptotic effectors (BAX and BAK), and anti-apoptotic inhibitors (such as BCL-2, BCL-XL, MCL-1 and BCL-W) (Cheng et al., 2001, Zong et al., 2001, Wei et al., 2001).

Among the anti-apoptotic BCL-2 family proteins, MCL-1 is distinctive because it represent the only anti-apoptotic BCL-2 family member with an embryonic lethal phenotype present in gene-deficient mice (Rinkenberger et al., 2000). MCL-1 is essential
for neuronal development as loss of MCL-1 in neuronal progenitors results in neuronal apoptosis (Arbour et al., 2008). Moreover, conditional MCL-1 gene deletion in the adult forebrain leads to massive cortical apoptosis and autophagy activation, suggesting a crucial role for MCL-1 also in the adult CNS (Germain et al., 2011). Interestingly, a recent study suggested that MCL-1 resides in two distinct isoforms and in two different mitochondrial compartments: an outer mitochondrial membrane localized MCL-1 that antagonises apoptosis by binding pro-apoptotic BCL-2 family members, and a mitochondrial matrix MCL-1 that increases mitochondrial membrane potential, respiration and ATP production, and regulates mitochondrial morphology (Perciavalle et al., 2012).

Preconditioning is a phenomenon where a stressful but not damaging stimulus activates an endogenous adaptive response to reduce the impact of subsequent, more severe stimuli. Preconditioning-induced neuroprotection with metabolic toxins or low-dose excitotoxic stimuli has been successfully employed in models of excitotoxic and ischemic injury (Wiegand et al., 1999, Navon et al., 2012, Smith et al., 2009). We have recently shown that mitochondrial uncoupling induced preconditioning protects neurons against subsequent excitotoxic injury through the activation of the energy sensor AMPK which mediates an improvement of cellular and mitochondrial bioenergetics (Weisova et al., 2012). While the mammalian kinase AMPK regulates many aspects of cellular energy status (Hawley et al., 1996, Stein et al., 2000), the molecular factors mediating AMPK-induced preconditioning in neurons are less well characterised. In this study, we investigate the molecular signalling pathways involved in AMPK-mediated
preconditioning induced by the pharmacological AMPK activator, AICAR, and identify MCL-1 as a key effector of AMPK-induced preconditioning.
Materials and Methods

Materials

Fetal bovine serum, horse serum, minimal essential medium (MEM), B27 supplemented Neurobasal media, Fluo-4 AM and tetramethylrhodamine methyl ester (TMRM) were from Invitrogen (Bio Sciences, Dublin, Ireland). 5-aminoimidazole-4-carboxamide riboside (AICAR) was obtained from Cell Signaling. Compound C (water soluble) was obtained from Calbiochem (Merck Biosciences, UK). Glutamate, glycine, MK-801 and all other chemicals were from Sigma-Aldrich.

Preparation of primary mouse neocortical neurons and cell culture

Mouse neocortical neurons were prepared and cultured as described previously (Concannon et al., 2010) with minor modifications. Briefly, the neocortex was isolated from embryonic day 16-18 mice by hysterectomy of pregnant female mice using 40 mg/kg of pentobarbital as lethal anesthesia. Isolated cortices were transferred to dissection medium on ice (PBS with 0.25% glucose and 0.3% BSA). The tissue was incubated in 0.25% trypsin-EDTA for 15 min. After the incubation, the trypsinization was stopped using media containing sera. The neurons were dissociated by gently pipetting and centrifugated at 300 g for 3 min and media containing trypsin was aspirated. Neocortical neurons were triturated in fresh plating medium (MEM containing 5% fetal bovine serum, 5% horse serum, 100 U/ml penicillin/streptomycin, 0.5 mM L-glutamine and 0.6% D-glucose). Cells were plated on poly-D-lysine-coated plates or Wilco dishes at 2 x 10^5 cells/cm^2 density, and maintained at 37 °C in a humidified atmosphere of 5% CO_2 / 95% air. Next day the plating medium was exchanged for 1:1 plating medium and
feeding medium (Neurobasal media containing 100 U/ml penicillin/streptomycin, 2%
B27 and 0.5 mM L-glutamine) containing 600 nM cytosine arabinofuranoside. On DIV 3
medium containing cytosine arabinofuranoside was exchanged for fresh feeding media.
All experiments were performed at DIV 9-11. All animal work was performed with
ethical approval from the RCSI and license granted by the Irish Department of Health and
Children. The motoneuron like cell line NSC-34 was grown in DMEM (Lonza)
supplemented with 10% fetal bovine serum, 2 mM L-glutamine and 100 U/ml
penicillin/streptomycin.

**NMDA toxicity and determination of neuronal injury**

Cortical neurons cultured on 24 well plate for DIV 9-11 were excited with
NMDA/glycine (100 μM/10 μM) for 5 min and washed twice in experimental buffer
containing (in mM): 120 NaCl, 3.5 KCl, 0.4 KH₂PO₄, 20 HEPES, 5 NaHCO₃, 1.2
Na₂SO₄, 1.2 CaCl₂ and 15 glucose, pH 7.4, supplemented with high Mg²⁺ (1.2 mM). To
assess neuronal injury resulting from NMDA excitation, neocortical neurons were stained
live with Hoechst 33258 at final concentration of 1 μg/ml after 24 h for 10 min at 37 °C.
After incubation, nuclear morphology was assessed using an Eclipse TE 300 inverted
microscope (Nikon) and 20x NA 0.45 dry objective. Images were taken using a
SPOT RT SE CCD camera and the appropriate filter sets. For each time point and
treatment, cells were scored for condensed nuclei as a marker of apoptotic morphology in
three subfields of each culture and repeated in triplicate. Condensed nuclei were counted
as dead and expressed as percentage of total population. Images were processed using
NIH Image J (Wayne Rasband, National Institute of Health, Bethesda, MD).
Gene expression analysis using real-time RT-PCR analysis

Total RNA was extracted using RNeasy mini kit (Qiagen). First strand cDNA synthesis was performed using 2 µg of total RNA as template and reverse transcribed using Superscript II (Invitrogen) primed with 50 pmol random hexamers. Real-time qPCR was performed using the LightCycler 2.0 (Roche) and QantiTech SYBR green PCR kit (Qiagen) according to manufacturer’s instruction. Each primer was specifically designed using Primer software (http://frodo.wi.mit.edu/). Sense and antisense primers are as follows: 5'-GCTCCGGAACTGGACATTA-3' and 5'-GTCCCGTTTTCGTCTTACAA-3' for MCL-1; 5'-AGCCATCCAGGCTGTGTTGTT-3' and 5'-CAGCTGTGGTGGTGAAAGCTG-3' for β-actin. The data were analysed using Lightcycler software 4.0 with all samples normalised to β-actin. The generation of specific PCR product was confirmed by melting curve analysis.

Protein extraction and Western blotting

Neocortical neurons cultured on 6 well dishes were mechanically extracted on ice. Whole cell extracts were obtained by centrifugation at 300 g for 3 min followed by wash with PBS. The cell pellets were lysed in ice-cold RIPA buffer (25 mM Tris HCl, 150 mM NaCl, 1% NP40, 1% sodium deoxycholate and 0.1% SDS). The cell lysates were spun at 300 g for 3 min and supernatants were used for Western blotting. Semi-dry transfer at 1.5 mA/cm² (approx. 75 mA/gel) for 90 min was used to transfer the protein from gel to nitrocellulose membrane (Schleicher and Schuell, BRD-100-520C). The resultant blots were blocked with 5% milk for an hour and probed with a rabbit polyclonal anti-phosphorylated-(Thr-172)-AMPK (Cell Signaling, 1:1000), a rabbit polyclonal total anti-
AMPKα antibody (Cell Signaling, 1:1000), a rabbit polyclonal anti-MCL-1 (Rockland, 1:1000), or a mouse monoclonal anti-β-actin (Sigma, 1:5000). Species specific horseradish peroxidase-conjugated secondary antibodies (Pierce, 1:10000) were detected using Super-Signal West Pico Chemiluminescent Substrate (Pierce) and imaged using a FujiFilm LAS-3000 imaging system (FujiFilm).

**Flow cytometry-based quantification of glucose transporter 3 (GLUT 3) cell surface expression**

Neocortical neurons cultured on 24 well plates were harvested after appropriate treatment and washed with PBS. Neurons were fixed with 1% formalin for 20-25 min on ice and quenched using glycine (0.1 M) and incubated in an anti- GLUT 3 antibody (Millipore Biosciences Research Reagents) at a concentration of 1:250 diluted in PBS plus 0.1% BSA at 4 °C overnight. Cells were washed twice with PBS and incubated with in an Alexa Fluor 488 goat anti-rabbit IgG (H+L) antibody (Invitrogen) diluted 1:250 for 1 h. Samples were acquired on a Partec CyFlow ML flow cytometer with a minimum of 10,000 events per sample and analysed using FloMax software.

**ATP luciferase assay**

Following treatment neurons were lysed using hypotonic lysis buffer (Tris-acetate buffer, pH 7.75). Levels of ATP were measured using luciferin-luciferase reaction kit (ENLITEN ATP Assay System Bioluminescence, Promega) as per manufacturer’s instruction. The amount of ATP was determined by a concentration standard curve and normalised to protein concentration corresponding to each sample and expressed as pmol ATP/mg protein. Data are presented as normalized to the vehicle treated controls.
Plasmids and transfection

Neocortical neurons (DIV 7) and NSC 34 cells were transfected using Lipofectamine 2000 (Invitrogen) or calcium-phosphate (Goetze et al., 2004). For inhibition of AMPK, cells were transfected with a vector expressing a siRNA targeting AMPK –α1/ α2 or a control sequence as previously described (Weisova et. al., 2009). For overexpression of MCL-1, cells were transfected with vector expressing MCL-1 (MC200829; OriGene). For inhibition of Mcl-1 cells were transfected with a vector expressing shRNA targeting MCL-1 (SCHLNG-Nm_008562; Sigma) or a scramble control vector (SHC001; Sigma). A plasmid with enhanced CFP (ECFP-C1; Clontech) was used to identify the transfected neurons for confocal microscopy experiments and a plasmid with enhanced GFP (eGFP-N1; Clontech) was used to allow the identification of transfected neurons for cell death assays. Cells were used for experiments 48 h after transfection.

Confocal microscopy

Primary neocortical neurons were loaded with TMRM (20 nM) and Fluo-4 AM (3 μM) for 30 min at 37 °C (in the dark) in experimental buffer. The buffer on Willco dishes (Willco Wells B.V., Amsterdam, NL) with neurons was exchanged for buffer without Fluo-4 AM, covered with a thin layer of embryo tested mineral oil (Sigma, Ireland) to prevent evaporation, and placed on a stage of an LSM 710 Zeiss confocal microscope with a 63x NA 1.4 differential interference contrast objective and a thermostatically regulated chamber maintained at 37°C. During live cell imaging multiple fields of view were addressed using the multiple time series macro in combination with the hardware autofocus. On stage cells were treated with 100 μM NMDA/10 μM glycine for 5 min.
Images were captured every 60 s. In order to terminate NMDA receptor activation, MK 801 (10 μM) was added to neurons 5 min after excitation. CFP was excited at 405 nm and the emission collected in the range of 440-490 nm, Fluo-4 was excited at 488 nm and the emission was collected in the range of 500-550 nm and TMRM was excited at 543 nm and emission was collected in the range of 560-700 nm. Images were processed using MetaMorph software version 7.5 (Molecular Devices, UK), and data were normalised to baseline response.

**Statistical analysis**

Data are presented as mean ± SEM. One-way ANOVA followed by Tukey’s *post hoc* test was performed to determine statistical significance. p values p≤0.05 were considered to be statistically significant.
Results

Preconditioning with a low concentration of AICAR activates AMPK and improves cellular bioenergetics

AICAR is a cell permeable precursor of the monophosphate nucleotide ZMP (Sabina et al., 1985), which mimics the effects of AMP on AMPK activation (Sullivan et al., 1994). Previous studies have established that AMPK can be activated using AICAR (Sabina et al., 1985, Sullivan et al., 1994), however, prolonged treatments with high concentrations of AICAR (1 – 5 mM) have also shown to promote apoptosis in neurons and other cell types (Concannon et al., 2010, Kefas et al., 2003, Meisse et al., 2002) similar to overexpression of constitutively active mutants of AMPK (Concannon et al., 2010, Meisse et al., 2002). We therefore set out to establish the optimal concentration of AICAR required to activate AMPK in cultured neocortical neurons without inducing cell death per se. Continuous treatment of neuronal cultures with different concentration of AICAR (0.1, 0.25, 0.5, 1.0 and 2.5 mM) for 24 hours resulted in a concentration dependent cell death as evidenced by nuclear pyknosis, with 0.1 mM AICAR showing no significant increase in toxicity over a 24 h time period (Fig. 1A). Maximal AMPK activation was observed after 2 h treatment with 0.1 mM AICAR (Fig. 1B, C), as determined by Western blot analysis using a specific antibody that detects activated, Thr172-phosphorylated AMPK (Hawley et al., 1996, Stein et al., 2000). This data suggested that treatment with AICAR at 0.1 mM activates AMPK in cultured neocortical neurons without any significant cytotoxic effects.
Previous studies have shown that AMPK activation is able to improve survival and cellular bioenergetics in primary neurons in response to glucose withdrawal or ATP depletion (Culmsee et al., 2001, Weisova et al., 2012). We therefore next investigated whether a 2 h exposure to 0.1 mM AICAR followed by wash-out improved cellular bioenergetics. A treatment with AICAR (0.1 mM) for 2 h resulted in a robust activation of AMPK (Fig. 2A, B) that was maintained for up to 24 h after wash-out (Fig. 2A, B). We have recently shown that activation of AMPK regulates glucose transporter 3 (GLUT 3) surface expression leading to neuronal tolerance during glutamate excitotoxicity (Weisova et al., 2009). Flow cytometry analysis demonstrated increased cell surface expression of GLUT 3 in neurons treated with 0.1 mM AICAR for 2 h, which remained elevated for up to 24 h after washout of AICAR (Fig. 2C). Similarly, the ATP levels of neurons treated with 0.1 mM AICAR for 2 h were significantly elevated up to 24 h post wash-out (Fig. 2D). Exposure to the protonophore FCCP which dissipates ATP production in mitochondria served as an internal negative control (Fig. 2D).

**Preconditioning with a low concentration of AICAR protects cortical neurons against NMDA induced excitotoxic cell death**

Previous studies have shown that AMPK activation prior to a toxic challenge is able to protect neurons against metabolic, excitotoxic and ischemic injury (Culmsee et al., 2001, Weisova et al., 2009, Weisova et al., 2012). We therefore next investigated whether ‘preconditioning’ of neurons induced by a 2 h exposure to 0.1 mM AICAR followed by washout protected primary cortical neurons in a well-characterized model of NMDA-induced excitotoxic injury (D'Orsi et al., 2012). Neurons were pre-treated with AICAR for 2 h, followed by washout and recovery for varying time periods up to 48 h before
being challenged with the NMDA stimulus (100 μM NMDA /10 μM glycine). As shown in Fig. 3, AICAR pre-treatment followed by washout and recovery for 24 h offered a significant protection against NMDA excitotoxicity in neurons exposed to NMDA for 5 min, as assessed by quantification of pyknotic nuclei using Hoechst 33258 staining 24 h after the NMDA exposure (Fig. 3A, B). No significant protection against NMDA excitotoxicity was observed after a 48 h washout period. These results suggested that activation of AMPK with low AICAR concentration (0.1 mM for 2 h) effectively pre-conditioned cortical neurons against NMDA excitotoxicity.

**Inhibition of AMPK activation abolishes AICAR preconditioning**

To demonstrate that AICAR preconditioning was mediated by an increased AMPK activity, we next employed the pharmacological AMPK inhibitor Compound C (Dasgupta and Milbrandt, 2007). Compound C was added 30 min before and during the AICAR treatment. Inhibition of AICAR-induced AMPK activation was evident in cortical neurons as determined by Western blotting (Fig. 4A). Inhibition of AMPK activity with Compound C also resulted in decreased GLUT 3 cell surface expression and ATP availability after 2 h of AICAR treatment (0.1 mM) (Fig. 4B, C). In addition, no significant neuroprotection against NMDA excitotoxicity was observed in neuronal cultures pre-treated with AICAR and Compound C for 2 h compared with cultures pre-treated with AICAR only for 2 h (Fig. 4D). Treatment with Compound C alone didn’t alter cell viability (data not shown).

To rule out potential off-target effects of pharmacological inhibition of AMPK with Compound C, we also investigated the effect of AMPK inhibition using gene silencing. Transfection of cortical neurons with a previously described siRNA targeting *AMPKa1*
(Weisova et al., 2009) (Fig. 5A) showed a significant increase in NMDA-induced cell
death in response to AICAR preconditioning when compared with control siRNA
transfected neurons (Fig. 5B). In agreement with previous studies showing pro-apoptotic
activities of prolonged AMPK activation during excitotoxic injury (Concannon et al.,
2010, Davila et al., 2012) there was a significant decrease in excitotoxic cell death in
AMPK siRNA transfected neurons that were not pre-conditioned with AICAR when
compared to neurons transfected with control siRNA (Fig. 5B). In summary, these results
suggest that AMPK activation was pivotal in mediating the effects of AICAR
preconditioning.

AICAR preconditioning increases mRNA and protein levels of the anti-apoptotic
BCL-2 family member MCL-1

To assess the role of increased gene transcription in mediating AICAR preconditioning-
induced protection against NMDA toxicity, we analysed the mRNA expression of several
genes involved in the regulation of cellular bioenergetics, mitochondrial function and in
the control of neuronal apoptosis, including mTFA, PGC-1α, BCL-w, survivin and MCL-
1 by quantitative PCR (qPCR). While mRNA levels of mTFA, PGC-1α, BCL-w and
survivin did not change significantly (data not shown); we detected increased mRNA
levels of the anti-apoptotic BCL-2 family protein, MCL-1. A significant increase in
MCL-1 mRNA was sustained up to 6 h after wash-out of AICAR (Fig. 6A). Western blot
analysis also revealed a significant increase in the protein levels of MCL-1 in response to
AICAR preconditioning, with similar kinetics (Fig. 6B). Furthermore, the AICAR-
induced increase in MCL-1 mRNA levels was abolished in neurons transfected with
AMPK siRNA compared to control siRNA transfected neurons (Fig. 6C). These results
suggest that AICAR preconditioning up-regulates the anti-apoptotic BCL-2 family protein MCL-1 in an AMPK-dependent manner.

**MCL-1 is neuroprotective against NMDA toxicity**

Previous studies demonstrated that overexpression of the anti-apoptotic proteins BCL-2 or BCL-XL can protect primary neurons against glutamate receptor-mediated excitotoxic injury (Dietz et al., 2007, Wang et al., 2004). We sought to determine whether MCL-1 exerts a similar neuroprotective activity in our setting of NMDA excitotoxicity. Neurons were transfected with a mammalian expression vector expressing MCL-1 and co-transfected with an EGFP expressing plasmid, or only transfected with the EGFP vector (controls). After 48 h cells were preconditioned with AICAR or treated with vehicle for 2 h, and then subjected to NMDA toxicity. Cell death of EGFP-positive cells was analysed 24 h after NMDA toxicity by evaluating nuclear pyknosis using Hoechst 33258 as well as cell shrinkage as morphological criteria (Fig. 7 A, B). Analysis of EGFP-positive neurons that were not pre-conditioned with AICAR indicated that transfection with MCL-1 significantly reduced cell death compared to EGFP-transfected neurons (Fig. 7C) There was no significant difference in protection achieved with AICAR preconditioning, MCL-1 overexpression, or combined AICAR preconditioning and Mcl-1 overexpression (Fig. 7C).

We next sought to determine whether AICAR preconditioning still exerted a protective activity in neurons silenced for MCL-1 expression. A shRNA construct targeting MCL-1 was successfully tested in murine NSC-34 cells and demonstrated strongly reduced MCL-1 protein levels after 48 h of transfection when compared with scrambled vector transfected cells (Fig. 7D). Transfection of the shRNA targeting MCL-1 in cortical
neurons induced significant cell death in control cultures when compared to neurons transfected with a scrambled vector (Fig. 7D), in line with previous findings demonstrating that MCL-1 is necessary to maintain survival in differentiated neurons (Germain et al., 2011). In addition, a significant increase in cell death was evident in NMDA-exposed neurons transfected with shRNA targeting MCL-1 when compared to scrambled vector transfected neurons. Of note, AICAR preconditioning failed to provide any protection in NMDA-exposed neurons transfected with MCL-1 shRNA when compared to non-preconditioned neurons transfected with either MCL-1 or scrambled shRNA (Fig. 7D). As expected, AICAR preconditioned neurons transfected with scrambled shRNA showed significant neuroprotection compared with non-preconditioned neurons. Collectively, these data suggest that MCL-1 is neuroprotective, and that MCL-1 gene silencing is dominant over the protective effect of AICAR preconditioning.

**AICAR preconditioning and MCL-1 overexpression reduce cytosolic Ca$^{2+}$ overloading during acute NMDA excitation**

Interestingly, both AMPK activation as well as MCL-1 overexpression has been shown to improve mitochondrial bioenergetics (Dasgupta and Milbrandt, 2007, Yu and Yang, 2010, Perciavalle et al., 2012). Since NMDA-induced Ca$^{2+}$ overloading is critically dependent on the bioenergetic ability of neurons to restore ion homeostasis (Weisova et al., 2009, Budd and Nicholls, 1998, Ward et al., 2000, Ward et al., 2007), we addressed the question whether AICAR preconditioning or MCL-1 overexpression is able to reduce NMDA-induced Ca$^{2+}$ elevations. To address this question, we performed time-lapse confocal imaging in cortical neurons co-loaded with Fluo-4 AM and TMRM in order to
determine the individual Ca$^{2+}$ and mitochondrial membrane potential responses to an acute NMDA challenge. Analysis of neurons transfected with a plasmid overexpressing MCL-1 revealed significantly reduced cytosolic Ca$^{2+}$ levels in response to NMDA compared to control transfected cells (Fig. 8A-C). Quantification of individual Ca$^{2+}$ responses revealed a marked decrease of cytosolic Ca$^{2+}$ influx in neurons with MCL-1 overexpression in comparison to control transfected neurons (Fig. 8C). Although MCL-1 overexpressing cells had a higher baseline TMRM fluorescence (Fig. 8D), no significant difference in the absolute depolarisation of $\Delta \psi_m$ was evident during NMDA excitation in neurons overexpressing MCL-1 compared with control-transfected neurons (Fig. 8E). Similar to MCL-1 overexpression, AICAR preconditioning significantly decreased cytosolic Ca$^{2+}$ levels in response to NMDA excitation. However, there was no significant difference observed between AICAR-preconditioned neurons and neurons overexpressing MCL-1 or AICAR-preconditioned neurons overexpressing MCL-1 (Fig. 8C). These data suggest that MCL-1 and AICAR preconditioning stabilizes neuronal Ca$^{2+}$ homeostasis in response to NMDA excitation.
Discussion

In the present study we describe a potent preconditioning effect resulting from a transient, 2-hour exposure to the AMPK activator, AICAR in a model of NMDA excitotoxicity in mouse cortical neurons. Interestingly, the preconditioning effect of the transient AICAR exposure was maintained for up to 24 h after wash-out of AICAR, suggesting that AICAR preconditioning likely induced long-lasting alterations in neurons. In line with this observation, we demonstrate that AICAR preconditioning was associated with increased MCL-1 mRNA and protein levels. Furthermore we demonstrate that MCL-1 exerts potent protective effects against NMDA-induced Ca\(^{2+}\) overloading and cell death, and that MCL-1 modulation is dominant over the effect of AICAR preconditioning. These experiments therefore identify the anti-apoptotic protein MCL-1 as a key effector of AMPK-mediated preconditioning in neurons.

Preconditioning has been extensively investigated in both in vitro and in vivo models of neuronal injury, and has been shown to protect neurons against excitotoxic, ischemic and oxidative injury (Kitagawa et al., 1990, Chen et al., 1996, Grabb and Choi, 1999, Navon et al., 2012). Several factors have been associated with the protective effect of preconditioning: these include the activation of potassium channels (Smith et al., 2009), maintenance of intracellular Ca\(^{2+}\) homeostasis (Bickler et al., 2010), preservation of mitochondrial membrane integrity, membrane potential, membrane fluidity as well as prevention of mitochondrial swelling (Zhang et al., 2003), activation of cyclic AMP response element-binding protein (Meller et al., 2005) and reduced activation of caspase 3, poly (ADP-ribose) polymerase, or nitric oxide synthase (Gidday et al., 1999, McLaughlin et al., 2003, Garnier et al., 2003). We have recently identified a key role for
the ancient energy sensor AMPK during preconditioning in neurons (Weisova et al., 2012). AMPK is expressed at high levels in neurons of the central nervous system (Turnley et al., 1999), and has previously been described to have potent neuroprotective effects (Culmsee et al., 2001). While AMPK is known to have numerous acute effects on mitochondrial function and cellular bioenergetics (Bergeron et al., 2001, Zong et al., 2002, Ojuka, 2004), key effectors of AMPK that may modulate long-term alterations in neurons have been less frequently described. We here identify the anti-apoptotic protein MCL-1 as a potential effector of AMPK that mediates some of AMPK’s preconditioning activities. Because MCL-1 mRNA and protein were found to be up-regulated up to 6 h after washout of AICAR, but neuroprotection was evident for up to 24 h after washout, other long-term alterations may also contribute to AMPK-induced preconditioning. In this context, AMPK has previously been shown to protect endothelial cells from hypoxia and oxygen glucose deprivation by up-regulating the anti-apoptotic proteins Bcl-2 and survivin (Liu et al., 2010). Our finding that MCL-1 is up-regulated at the mRNA and protein level is interesting since MCL-1 is subject to rapid protein turnover due to proteasomal degradation (Nijhawan et al., 2003, Akgul et al., 2000, Yang et al., 1995), and considering that AMPK activation inhibits cap-dependent protein translation (Liu et al., 2006, Kimura et al., 2003). The mechanisms responsible for the MCL-1 mRNA up-regulation in response to AICAR therefore warrant further investigation, and may include effects of AMPK on mRNA stability or proteasomal degradation as shown in other systems previously (Viana et al., 2008, Yun et al., 2005).
MCL-1 has previously been suggested to be a key regulator of neuronal survival by inhibiting apoptosis and by inhibiting a potentially toxic overactivation of autophagy (Germain et al., 2011). MCL-1 has also been shown to be essential for neuronal development, as deletion of MCL-1 induced apoptosis in neuronal progenitors and newly committed neurons as they commence their migration away from the ventricular zone (Arbour et al., 2008). Recently, MCL-1 has been shown to regulate the survival of adult neuronal precursors cells, with conditional deletion or over expression of MCL-1 increasing or decreasing neuronal apoptosis by two fold respectively (Malone et al., 2012). However, the effect of MCL-1 may extend beyond the regulation of the mitochondrial apoptosis pathway. Indeed, a recent study has demonstrated that MCL-1 exists in two forms that regulate apparently different aspects of mitochondrial function: MCL-1^{OM} (40 and 38 kDa) and MCL-1^{Matrix} (36 kDa). MCL-1^{OM} resides on the mitochondrial outer membrane and antagonizes pro-apoptotic proteins to inhibit mitochondrial outer membrane permeabilization. In contrast, MCL-1^{Matrix} localizes to the matrix and maintains normal inner mitochondrial membrane structure, and increases mitochondrial membrane potential and bioenergetics (Perciavalle et al., 2012) Of note, we noted a significant increase in the protein levels of both MCL-1 variants in response to AICAR preconditioning, suggesting that these are jointly up-regulated by AMPK. The finding that AMPK also up-regulated the protein levels of MCL-1^{Matrix} is particularly interesting considering the ancient function of AMPK in the maintenance of cellular bioenergetics. Improved mitochondrial function and bioenergetics are known to facilitate the extrusion of Ca^{2+} during Ca^{2+} overloading, by activating plasma membrane Ca^{2+}-ATPases and Na^{+}/Ca^{2+} exchangers (White and Reynolds, 1995, Kiedrowski and Costa,
1995). We observed significantly reduced cytosolic Ca\textsuperscript{2+} levels in response to NMDA in MCL-1-overexpressing and AICAR-preconditioned cells. It is therefore likely that neuroprotection induced by either MCL-1-overexpression or AICAR preconditioning is largely due to a significantly reduced neuronal Ca\textsuperscript{2+} overload during the initial period of NMDA excitation. Our finding that MCL-1 overexpression itself reduces cytosolic Ca\textsuperscript{2+} overloading may further indicate that MCL-1 is indeed an evolutionary conserved regulator of mitochondrial physiology, similar to other BCL-2 family proteins and apoptosis-associated proteins for which such ‘day-time’ roles have been increasingly described (Kilbride and Prehn, 2012).

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The authors declare no conflict of interest.

Reference


CULMSEE, C., MONNIG, J., KEMP, B. E. & MATTSON, M. P. 2001. AMP-activated protein kinase is highly expressed in neurons in the developing rat brain and
promotes neuronal survival following glucose deprivation. *J Mol Neurosci.*, 17, 45-58.


MCL-1 localizes to the mitochondrial matrix and couples mitochondrial fusion to respiration. *Nat Cell Biol.*


WEISOVA, P., CONCANNON, C. G., DEVOCELLE, M., PREHN, J. H. & WARD, M. W. 2009. Regulation of glucose transporter 3 surface expression by the AMP-
activated protein kinase mediates tolerance to glutamate excitation in neurons. *J Neurosci*, 29, 2997-3008.


Figure 1: Activation of AMPK with a subtoxic concentration of AICAR (0.1 mM). A) Dose-response study in cortical neurons. Nuclei were stained with Hoechst 33358 (1 μg/ml) 24 h after AICAR addition. Controls were treated with vehicle. Neurons with uniformly stained nuclei were counted as viable and condensed nuclei counted as apoptotic. Experiments were repeated twice in triplicate treatments, *p≤0.05, difference between vehicle and 0.25 to 2.5 mM AICAR treatment. B) Treatment with AICAR (0.1 mM) activates AMPK as analyzed by Western blotting. β-actin served as loading control. Similar responses were obtained in samples from two separate cultures (C) AMPK activation quantified by densitometry of Western blotting experiments and normalized to total AMPK levels. *p≤0.05, difference between vehicle and AICAR treated at 2 h and 4 h. Data are presented as means ± SEM (n = 4 treatments from two platings).

Figure 2: AICAR (0.1 mM) upregulates AMPK and stimulates neuronal metabolism in murine cortical neurons. A) Model of AICAR preconditioning. Neurons were treated with 0.1 mM AICAR for 2 h, the medium was exchanged, and cells were recovered for various time periods up to 48 h after washout (Upper panel). Western blot analysis of AMPK activation status in cultured cortical neurons treated with AICAR (0.1 mM) for 2 h and washout for the indicated time periods (Lower panel). B) Densitometry analysis showing activated AMPK normalized to total AMPK activity and expressed relative to vehicle treated controls. *p≤0.05, difference between vehicle and AICAR treated neuronal cultures. Similar responses were obtained from three separate cultures. C) Quantification of GLUT 3 surface expression by flow cytometry. Data are represented as
mean ± SEM. *p<0.05, differences between vehicle and AICAR treated neurons. Experiments were performed in triplicate in three separate cultures. D) Cortical neurons were treated with AICAR and their ATP content measured (pmol ATP/mg protein) at indicated time points. n = 2 experiments in triplicate; *p<0.05, difference between vehicle and AICAR treated neurons. Data reported as mean ± SEM.

Figure 3. AICAR pretreatment protects cortical neurons against NMDA-mediated excitotoxicity. A) Representative images of Hoechst-stained neurons treated with vehicle or AICAR, or exposed to NMDA /glycine (100 μM/10μM) for 5 min in the absence or presence of AICAR preconditioning. Images were taken 24 h post NMDA excitation. Scale bar, 10 μm. B) Quantification of cell death. Neuronal cultures were treated with AICAR (0.1 mM) for 2 h and subsequently exposed to NMDA at 0 h, 6 h, 24 h and 48 h post AICAR washout time points. Nuclei were stained with Hoechst and uniformly stained nuclei counted as viable and condensed nuclei scored as dead. Data are presented as mean ± SEM. *p<0.05, difference between NMDA treated and AICAR washout treated with NMDA (n = 9 treatments performed on neurons from three different platings).

Figure 4: Inhibition of AMPK activation with Compound C abolishes the effect of AICAR preconditioning on neuronal bioenergetics and NMDA toxicity. A) Western blot analysis of cortical neurons treated with AICAR (0.1 mM for 2 h) only or co-treated with Compound C (10 μM) and harvested immediately afterwards. B) Quantification of relative fluorescence intensity (arbitrary units) by flow cytometry for GLUT 3 surface expression. *p≤0.05, difference between neurons treated with AICAR (0.1 mM for 2 h) and those co-treated with Compound C (10 μM). Experiments were performed from three
separate cultures in triplicate. C) Cortical neurons were treated with AICAR (0.1 mM for 2 h) only or co-treated with Compound C (10 μM) and ATP content (pmol/mg protein) was measured by luminescence assay. Data shown represents mean ± SEM. *p<0.05, difference between neurons treated with AICAR and co-treated with Compound C. Experiments were repeated in triplicate with two independent culture preparation with similar results. D) Cortical neurons treated with AICAR alone or co-treated with Compound C and exposed to NMDA for 5 min and assayed over 24 h. Nuclei were stained with Hoechst, with uniformly stained nuclei counted as viable and condensed nuclei scored as dead. Data are presented as mean ± SEM. *p<0.05, compared with AICAR co-treated with Compound C and exposed to NMDA and NMDA only treatment. Experiments were performed in triplicate in two different cultures with similar results.

Figure 5. AMPK inhibition using gene silencing attenuates AMPK mediated neuroprotection against NMDA-induced excitotoxicity. A) Western blot analysis of NSC 34 cells transfected with Control siRNA or AMPK siRNA. AMPK levels were assessed 48 h after transfection by Western blotting. β-actin served as loading control. B) Cortical neurons were transfected with siRNA vector targeting AMPK a1/a2 or a control siRNA. Condensed nuclei in the transfected cells were quantified (n=120-148 cells per time point quantified). *p<0.05, compared with Control siRNA transfected neurons exposed to NMDA. #p≤0.05, difference between Control siRNA and AMPK siRNA transfected neurons treated with AICAR and exposed to NMDA. NS, no significance. Experiments were repeated three times in independent cultures with similar results. Data presented as mean ± SEM.
Figure 6: AICAR preconditioning increases mRNA and protein levels of Mcl-1 at the time point of maximal protection against NMDA excitotoxicity. A) Real time qPCR analysis of MCL-1 mRNA levels in cortical neurons treated with AICAR (0.1 mM for 2 h) with washout time points indicated. Expression levels were normalized to β-actin mRNA and expressed relative to vehicle treated control. Data shown represents mean ± SEM from n = 3 experiments. *p<0.05, difference between vehicle treated and AICAR treated cultures. B) Western blot and densitometric analysis of MCL-1 protein levels in cultured cortical neurons treated on DIV 9-11 with 0.1 mM AICAR for 2 h and washout as indicated. For quantification MCL-1 levels were normalized to β–actin. Data presented as mean ± SEM. *p<0.05, difference between vehicle and AICAR treated neurons from three separate cultures. C) Real time qPCR analysis of MCL-1 expression in cortical neurons transfected with control siRNA or AMPK siRNA and treated with vehicle or AICAR (0.1 mM for 2 h). Expression levels were normalized to β-actin and expressed relative to vehicle treated control. Data shown represents mean ± SEM from n = 3 experiments. *p<0.05, difference between vehicle treated and AICAR treated cultures in control siRNA transfected neurons.

Figure 7: Mcl-1 plays a critical role in AICAR induced neuroprotection following NMDA induced excitotoxicity in cortical neurons. A, B) Representative images of empty vector and Mcl-1 vector transfected cortical neurons. Cortical neurons were vehicle treated and sham-exposed (controls Vehicle), pre-treated with AICAR (0.1 mM for 2 h) and subsequently exposed to NMDA, or pre-treated with vehicle and then exposed to NMDA. GFP and Hoechst 33342 (1 μg/ml) fluorescence images were acquired to identify transfected cells and quantify nuclear apoptosis. Scale bar, 5 μm. C)
Quantification of the effect of MCL-1 overexpression (OE) on cell survival after NMDA toxicity. Neurons were vehicle treated and sham-exposed, pre-treated with AICAR (0.1 mM for 2 h) and subsequently exposed to NMDA, or pre-treated with vehicle and then exposed to NMDA. A total of n = 88-168 cells per treatment were quantified. Experiments were repeated in two independent cultures. *p≤0.05, compared with NMDA exposed, empty vector transfected controls. NS, no significance, compared between MCL-1 OE neurons exposed to NMDA treated with or without AICAR. D) Effect of MCL-1 gene silencing. Top: NSC34 cells were transfected with shRNA against MCL-1 or scramble shRNA. Reduced expression levels of MCL-1 after shRNA expression compared with scramble were assessed by Western blotting 48 h after transfection. β-actin served as control. Bottom: Quantification of neuronal survival. Neurons transfected with either MCL-1 shRNA or control shRNA were vehicle treated and sham-exposed, pre-treated with AICAR (0.1 mM for 2 h) and subsequently exposed to NMDA, or pre-treated with vehicle and then exposed to NMDA. A total of n = 98-126 cells per treatment condition were quantified. Experiments were performed in two independent cultures. *p≤0.05, differences between MCL-1 shRNA and Scramble shRNA transfected neurons. #p≤0.05, compared with NMDA exposed Scramble shRNA transfected neurons with or without AICAR preconditioning.

**Figure 8: Mcl-1 inhibits neuronal Ca^{2+} overloading during NMDA excitation.** A, B) Representative traces from cortical neurons transfected with a MCL-1 overexpressing vector or control empty vector for 48 h. Neurons were loaded with TMRM and Fluo-4 AM for 30 min at 37°C before being mounted on the stage of LSM710 and treated with 100 μM NMDA for 5 min. C) Quantification of Fluo-4 fluorescence (area under the
curve) in neurons transfected with MCL-1-overexpressing or empty vector. Neurons were pre-treated with AICAR or vehicle. Data were obtained from 6 separate experiments from 3 different cultures (n = 19-25 neurons per treatment quantified). *p≤0.05, compared with empty vector transfected neurons treated with NMDA only. NS, no significance. D) Quantification of baseline TMRM fluorescence in MCL-1 overexpressing or empty vector-transfected neurons. *p≤0.05, compared with empty vector transfected neurons. Two sample t-test assuming equal variances was used to assess the statistical significance. E) Quantification of NMDA-induced depolarization by TMRM fluorescence measurements in neurons transfected with MCL-1 or empty vector. NS, no significance, two sample t-test assuming equal variances was used to assess the statistical significance.
Figure 1

A. Cell death (%) with different AICAR concentrations.

B. Western blot showing p-AMPK (62 kDa), AMPK (62 kDa), and β-actin (42 kDa) levels with AICAR 0.1 mM treatment for 2 h, 4 h, 6 h, and 8 h.

C. p-AMPK (% of control) with 0.1 mM AICAR treatment for 2, 4, 6, and 8 hours.
Figure 4

A

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<td>![Image of blots for AMPK (62 kDa)]</td>
<td>![Image of blots for β-actin (42 kDa)]</td>
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B

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C

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D

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**Figure 5**

**Panel A**

![Image of Western Blot](image1)

**Panel B**

![Bar Graph](image2)

- **X-axis:** Vehicle, AICAR 2 h, NMDA, NMDA + AICAR 2 h
- **Y-axis:** Cell death (%)
- **Groups:** Control siRNA, AMPK siRNA

Legend:

- **Control siRNA**
- **AMPK siRNA**

Significance:

- *: p < 0.05
- #: p < 0.01
- NS: Not Significant
Figure 6

(A) MCL-1 mRNA

(B) Mcl-1 (% of control)

(C) MCL-1 mRNA

Vehicle 0 h      2 h      4 h      6 h     24 h    48 h
AICAR washout

MCL-1 (35 kDa)

β-actin (42 kDa)
Figure 7

(A) Empty vector transfected cells

(B) MCL-1 cDNA transfected cells

(C) Cell death (%)

(D) Protein expression of MCL-1 and β-actin

Legend:
- Empty vector
- MCL-1 OE
- Scramble shRNA
- MCL-1 shRNA

Notes:
- NS
- *Significant difference
- #Not significant
A

Control

![Graph showing Fluo-4 and TMRM fluorescence over time in NMDA and NMDA+AICAR conditions.](image)

B

MCL-1

![Graph showing Fluo-4 and TMRM fluorescence over time in Control and MCL-1 conditions.](image)

C

Area under the curve

![Bar chart showing Fluo-4 fluorescence in NMDA and NMDA+AICAR conditions](image)

D

![Bar chart showing TMRM fluorescence in Control and MCL-1 conditions](image)

E

![Bar chart showing TMRM fluorescence difference between Control and MCL-1 conditions](image)